Characterizing Oncogene Addiction in Alveolar Rhabdomyosarcoma to Find New Combination Therapies

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Zürich, 2018

To my parents.

HUMAN BEINGS MAKE LIFE SO INTERESTING. IN A UNIVERSE SO FULL OF WONDERS, THEY HAVE MANAGED TO INVENT BOREDOM.

- Sir Terry Pratchett

The experimental work presented in this thesis was performed at the Department of Oncology and Children's Research Center, University Children's Hospital Zurich. The supervision of the thesis was conducted by Prof. Dr. Beat W. Schäfer (Department of Oncology, University Children's Hospital Zurich), Prof. Dr. Alessandro Sartori (Institute of Molecular Cancer Research, University of Zurich), and PD Dr. Paolo Cinelli (Division of Trauma Surgery, Center for Clinical Research, University Hospital Zurich).

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1. SUMMARY

Pediatric cancer is the most common disease-related form of death in children and adolescents. The advent of chemotherapy and radiotherapy in the middle of the last century helped to drastically decrease the mortality rate. Yet, the harsh treatment regimens come at the price of developmental defects, cognitive impairments, and the increased risk of secondary malignancies even after years of the initial treatment. Despite the higher chances of curing patients, many cancers relapse and become resistant to the standard-of-care therapy. Thus, both to improve survival rates of patients and to reduce the burden of side-effects new treatment strategies need to be found, targeting cancer specific abnormalities. Many cancers become so reliant on specific oncogenic signals that withdrawal of these signals has detrimental effects on the cancer cells. This, so called oncogene addiction offers opportunities to target cancer cells pharmacologically.

One example of oncogene addiction can be seen in rhabdomyosarcoma (RMS), specifically the alveolar subtype. RMS is the most common soft-tissue sarcoma in children, accounting for 3-4% of all pediatric cancers. The two major subtypes, embryonal RMS (eRMS) and alveolar RMS (aRMS), have their peak of incidence in early childhood and adolescence, respectively. aRMS tumors have the worst prognosis of all childhood RMS cases with 5-year overall survival rates of only around 21%.

The pathognomonic aberration in aRMS is a chromosomal translocation. Most commonly this translocation results in the expression of the fusion protein PAX3-FOXO1. This fusion protein is a transcriptional activator and the major driver of aRMS tumorigenesis and essential for tumor cell survival. Thus, aRMS cells are addicted to the oncogenic features of this fusion protein. As targeting a transcription factor directly is near impossible, we wanted to find other strategies to interfere with the biology of the fusion protein.

Hence, our aim in this study was, on the one hand, to characterize the effectors by which PAX3-FOXO1 prevents cell death in aRMS cells. On the other hand, we aimed to find new ways to pharmacologically interfere with PAX3-FOXO1 stability. Based on these findings, the foremost aim was to find a combination therapy, priming aRMS cells to cell death resulting from loss of PAX3-FOXO1 activity.

Here, we demonstrate that aRMS cells upregulate the BH3-only protein NOXA and undergo intrinsic apoptosis dependent on NOXA, after genetic silencing of PAX3-FOXO1. In line with these findings we show that the BH3-mimetic ABT-263 (Navitoclax) can prime aRMS cells for this mode of cell death in NOXA-dependent manner. Furthermore, we demonstrate a novel functional interaction of PAX3-FOXO1 with Aurora kinase A which stabilizes the fusion protein by phosphorylation. Consequently, inhibition of Aurora kinase A, with the small molecule inhibitor Alisertib results in less PAX3-FOXO1 stability. In line with these findings, combination of both Navitoclax and Alisertib synergistically induces cell death in vitro and with lasting effects reduces xenograft tumor growth in vivo.

Lastly, we also established a potential novel transcriptional regulation of MDM2 by the fusion protein in aRMS. Consequently, we show that aRMS cells from patient-derived xenografts are sensitive to pharmacological disruption of the MDM2/p53 interaction by Idasanutlin.

Taken together, this study reveals new functional interactions of PAX3-FOXO1 and its activities to prevent cell death and provides novel strategies for combination therapies.

2. ZUSAMMENFASSUNG

Krebs ist die häufigste krankheitsbedingte Todesursache bei Kindern und Jugendlichen. Die Entwicklung von Chemotherapeutika und Strahlentherapie Mitte des vorherigen Jahrhunderts konnte die Sterblichkeitsrate drastisch senken. Dennoch führen die harschen Behandlungen bei Kindern zu vielen Nebenwirkungen, wie Entwicklungsstörungen, kognitiven Einschränkungen und einem erhöhten Risiko sekundäre maligne Erkrankungen zu entwickeln, selbst Jahre nach der ursprünglichen Behandlung. Trotz hoher Chancen, Patienten zu heilen, können viele Krebsarten resistent gegenüber der Standardbehandlung werden. Es müssen also neue Therapien entwickelt werden, die spezifisch gegen Krebszellen gerichtet sind, um die Überlebensraten zu steigern und gleichzeitig Nebenwirkungen zu reduzieren. Viele Krebszellen werden abhängig von onkogenen Signalen, dass der Entzug dieser Signale vernichtende Wirkungen auf die Zellen hat. Dieses Konzept der Onkogenabhängigkeit bietet neue Chancen, Krebszellen pharmakologisch anzugreifen.

Ein Beispiel der Onkogenabhängigkeit kann in Fällen des alveolären Rhabdomyosarkoms (RMS) beobachtet werden. Mit 3-4% aller pädiatrischen Krebsfälle ist RMS die häufigste Form des Weichteilsarkoms in Kindern. Die zwei Haupttypen sind das embryonale und das alveoläre RMS (aRMS). Das embryonale RMS tritt am Häufigsten im frühen Kindesalter auf, während die meisten aRMS-Fälle bei Jugendlichen auftreten. Mit einer 5-Jahres Überlebensrate von gerade einmal 21% haben aRMS Tumoren die schlechteste Prognose aller RMS Fälle.

Typisch für aRMS Tumoren ist, dass sie eine chromosomale Translokation aufweisen. Am Häufigsten führt diese dann zur Expression des Fusionsproteins PAX3-FOXO1. Dieses Fusionsprotein ist als Transkriptionsfaktor die treibende Kraft in der aRMS Tumorgenese und essentiell für das Überleben der Tumorzellen. Daher sind aRMS Zellen von diesem Onkogen abhängig. Leider ist sehr schwierig einen Transkriptionsfaktor pharmakologisch zu inhibieren. Daher wollen wir neue Strategien finden, wie man die Aktivität des Fusionsprotein unterdrücken kann.

Das Ziel dieser Studie war daher einerseits, die Effektoren zu charakterisieren, durch die PAX3-FOXO1 den Zelltod in aRMS Zellen verhindert. Weiterhin wollten wir neue pharmakologische Ansätze finden, die Stabilität des Fusionsproteins zu beeinflussen. Letztendlich war das Ziel, eine Kombinationstherapie zu finden, die aRMS Zellen für den Zelltod sensitivieren der aus dem Verlust von PAX3-FOXO1 resultiert.

In dieser Studie zeigen wir, dass aRMS Zellen das BH3-only Protein NOXA hochregulieren und in Apoptose gehen, wenn man das Fusionsprotein mit genetischen Mitteln ausschaltet. Der Wirkstoff ABT-263 (Navitoclax) sensitiviert aRMS Zellen für diese Form des Zelltods. Weiterhin zeigen wir eine neue funktionale Interaktion zwischen PAX3-FOXO1 und Aurora Kinase A, welche das Fusionsprotein durch Phosphorylierung stabilisiert. Inhibition von Aurora Kinase A mittels Alisertib reduziert konsequenterweise die Stabilität des Fusionsproteins. Die Kombination von Alisertib und Navitoclax induziert Zelltod in vitro und reduziert das Tumorwachstum stabil in Xenograft Experimenten in Mäusen. Zuletzt zeigen wir noch eine potentielle neue Regulation von MDM2 durch das Fusionsprotein und dass Zellen aus Patientenproben sensitiv gegenüber der Inhibition von MDM2 durch Idasanutlin sind.

Zusammengefasst zeigt diese Studie neue funktionelle Interaktionen von PAX3-FOXO1 auf und wie es Zelltod verhindert. Weiterhin zeigen wir neue Ansätze für Kombinationstherapien des aRMS.

3. LIST OF ABBREVIATIONS

ACD Accidental cell death
ADCC Antibody-dependent cellular cytotoxicity
ALL Acute lymphoblastic leukemia
APAF1 Apoptotic protease activating factor 1
ATM Ataxia telangiectasia mutated
ATP Adenosine triphosphate
ATR Ataxia telangiectasia and Rad3 related
BAK Bcl-2 homologous antagonist killer
BAX Bcl-2 associated X protein
BCL-2 B-cell lymphoma 2
BRAF Rapidly accelerated fibrosarcoma
BRCA1 Breast Cancer 1, early onset
CAR Chimeric antigen receptor
CASP Caspase
CHK Checkpoint kinase
CLL Chronic lymphatic leukemia
CML Chronic myelogenous leukemia
COG Children's Oncology Group
DAMP Danger associated molecular pattern
DDR DNA damage response
EGF Epidermal growth factor
EpSSG European pediatric Soft tissue sarcoma Study Group
ER Endoplasmic reticulum
FDA U.S. Food and Drug Administration
FGF Fibroblast growth factor
FOXO1 Forkhead box protein O1
HDAC Histone deacetylase
IGF Insulin-like growth factor
IRSG Intergroup Rhabdomyosarcoma Study Group
mAb Monoclonal antibody
Mb Mega base
MDM2 Mouse double minute 2 homolog
MOMP Mitochondrial outer membrane permeabilization
mTOR Mammalian target of rapamycin
OMM Outer mitochondrial membrane
PARP Poly (ADP-ribose) polymerase
PAX Paired box gene
PI3K Phosphoinositide 3 kinase
PLK1 Polo-like kinase 1
PMAIP1 Phorbol-12-myrisate-13-acetate-induced protein 1 (NOXA)
PPTP Pediatric Preclinical Testing Program
PS Phosphatidyl serine
RCD Regulated cell death
RMS Rhabdomyosarcoma
ROS Reactive oxygen species
SAC Spindle assembly checkpoint
VAC Vincristine / Actinomycin D / Cyclophosphamide
VEGF Vascular endothelial growth factor
WHO World Health Organization

4. INTRODUCTION

4.1 CANCER

4.1.1 General

Cancer as a disease has been reported throughout human history. The earliest written reports date back to around the year 3000 BC: On an ancient Egyptian papyrus a physician described the resection of a tumor in a female breast using a tool called “fire drill”, indicating a cauterizing method. The description concludes with the words: “There is no treatment”.[1] Later, Hippocrates names the disease *Καρκίνος* (karkinos). Literally translated this name means “crab”, reflecting the resemblance of the tumor and its veins to the animal dug in the sand with its legs spread around it.[1] Today, cancer is the one of the leading causes of death worldwide, second only to cardiovascular diseases.

However, cancer is not “one” disease, but rather a set of diseases sharing common characteristics: They are the result of a process in which a cell sequentially acquires multiple mutations activating oncogenes, e.g. BRAF[2], or inactivating tumor suppressor genes, e.g. TP53[3]. The altered function of these genes leads to dysregulation of proliferation, survival signals, and response to anti-growth signals.[4] Mutations in stability genes such as BRCA1, which is responsible for DNA damage repair, increase the mutation rate in a cell and contribute to tumorigenesis.[5] As cells acquire more mutations they divide more rapidly and progress from a histological hyperplasia, via dysplasia into a cancer in situ that loses its tissue architecture and features but is still confined by the basal membrane (**Figure 1**). Ultimately, through more mutations cancer cells acquire possibilities to breach the basal membrane, invade into the surrounding tissue and enter the blood stream or lymphatic vessels to seed into distant organs, where they form secondary tumors, called metastases.[5]

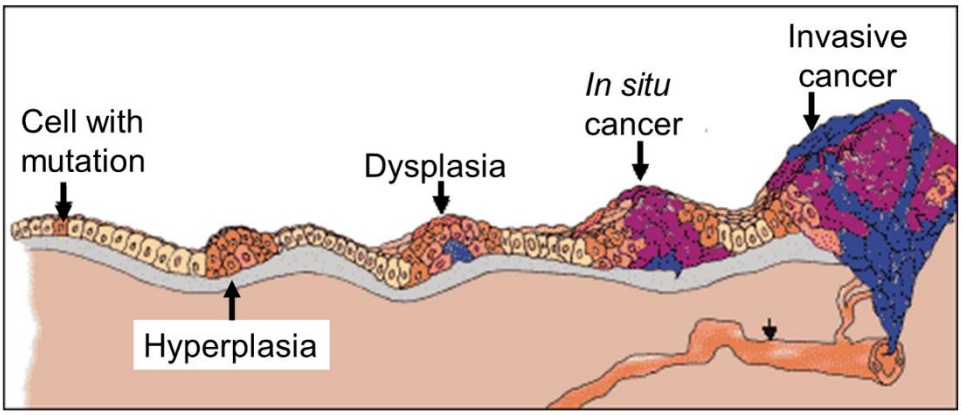

Figure 1: Stages of carcinogenesis from organized tissue to invasive and metastatic cancer. Adapted from[6]

In the year 2000, Douglas Hanahan and Robert Weinberg proposed a model of oncogenic traits that almost every cancer will acquire, irrespective of its origin.[7] These hallmarks of cancer are: resisting cell death, sustained proliferation, insensitivity to anti-growth signals, invasion and metastasis, replicative immortality and sustained angiogenesis. [7] After a revision in 2011, four new traits where added to the

hallmarks, acknowledging the interaction of cancer cells with their surrounding environment: evading immune destruction, tumor promoting inflammation, deregulating cellular energetics, and genome-instability (**Figure 2**).[8] While the exact sequence of when these alterations occur can differ, ultimately these hallmarks are common to almost all cancers and will dictate their malignancy (**Figure 2**).[7]

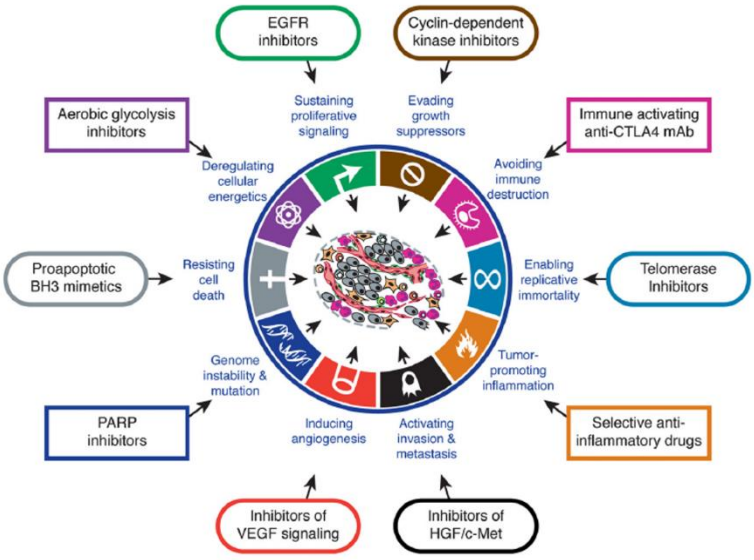

***Figure 2**: The hallmarks of cancer as proposed by Hanahan and Weinberg. This revised version also acknowledges the influence of the cancer on the microenvironment. The outer circle indicates therapeutic ways to target each hallmark. Adapted from[8]*

4.1.2 Classification

Tumors can be classified into benign (non-cancerous) and malignant tumors (cancerous). A benign tumor grows locally restricted within the boundaries of a basal membrane. Malignant tumors are those that breach the basal membrane, invade surrounding tissues, and metastasize.[9] These malignant cancers account for the majority of cancer-related deaths. Carcinomas are malignant tumors arising in the epithelium, while those originating in the connective and supporting tissues (i.e. of mesenchymal origin) are called sarcomas. Cancers of the hematological system are called leukemia or lymphomas, neuroectodermal cancer arises from cells of the nervous system.[4]

4.1.3 Epidemiology

In the adult population the most common cancers by far are carcinomas, especially those arising in lung, colon or stomach, as well as prostate in men and breast and cervix in women. In 2015 8.8 Million people worldwide died of cancer. Globally, this number accounts for 1/6 of all deaths.[10,11]

4.1.4 Etiology

Cancer is a genetic but not necessarily inherited disease, involving mutations or other damage at the DNA level.[2] As oncogenes, tumor suppressor genes, and stability genes are all encoded in the DNA, mutations in the gene or other genomic alterations can affect the expression and function of these genes. Herein lies the basis of tumorigenesis. Alterations are acquired throughout life, either intrinsically through sporadic mutation events or facilitated by extrinsic noxae. These extrinsic factors can be grouped into chemical agents (e.g. benzopyrene, aflatoxin), physical agents (i.e. ionizing and non-

ionizing radiation), and biological agents (viruses, bacteria). Furthermore, personal life style, e.g. smoking, diet, alcohol consumption, can contribute to cancer risk. Apart from these acquired alterations, mutations can also be inherited, which predisposes the patient to develop cancer. A single mutation does not turn a cell cancerous right away but rather provides a selective growth advantage.[5] Thus, the first mutation in either an oncogene or a tumor suppressor is considered a "gatekeeping" event. Over years, through the aforementioned factors, cells can acquire more mutations that further increase their proliferation rate or contribute to the tumors invasiveness.[5] The sequential acquisition of mutations explains why cancer risk increases with age.

4.1.5 Genomic and genetic alterations in cancer

Typical genetic aberrations in cancer are point mutations or insertion/deletion events, leading to crucial amino acid exchanges (e.g. BRAF V599E in melanoma[12]) or truncation of proteins (e.g. AR-V7 in prostate cancer[13]). In addition to mutations in the gene sequence, other alterations of the genome can contribute to tumorigenesis. Cancer cells also show elevated levels of chromosomal aberrations.[14] Large portions of chromosomes can be deleted or amplified, which results in loss of tumor suppressor genes or increased copy number of oncogenes, respectively. Copy number variations of oncogenes can range von 10-100 copies of one oncogene in one cell, compared to two copies in a normal cell.[5] Even whole chromosomes can be lost (aneuploidy) or duplicated (polyploidy) to a large extend in cancer cells.[15] Apart from these chromosomal anomalies, cancer cells can also harbor chromosomal translocations that lead to the fusion of two unrelated genes. In some cases, the result of such a translocation is a functional oncogenic fusion protein, for instance BCR-ABL in chronic myelogenous leukemia (CML) or PAX3-FOXO1 in rhabdomyosarcoma (see below).[16] In other cases, an oncogene is placed under the control of a new promotor or obtains new regulatory elements, for instance IG-MYC in Burkitt's lymphoma[17] or TMPRSS2-ERG in prostate cancer[18]. In the case of tumor suppressor genes, chromosomal translocations most often lead to the truncation and inactivation.[5]

4.1.6 Conventional therapy

4.1.6.1 Surgery and radiation

The major goal of cancer therapy was and is, to kill the tumor. Today, in most cases the standard therapy comprises surgery, chemotherapy, and radiation. Surgery, to remove the primary tumor, already performed in ancient Egypt.[1] For benign tumors, this method can be applicable and even curative. However, malignant tumors can infiltrate the surrounding tissue making it hard to remove the complete tumor mass. To prevent relapse, in the late 19th century, the American surgeon William Halsted performed radical mastectomies to make sure that the whole tumor was removed, albeit having infiltrated the tissue.[19] At that time this harsh treatment went hand in hand with a drastic decrease in quality of life of the patients, many of whom succumbed to the treatment rather than the disease. Moreover, the greatest risk of aggressive tumors comes from their ability to form metastases. In many cases multiple micro-metastases exist in the body, which are not visible by eye and thus impossible to resect. Surgery has its limits in conditions where the tumor mass occurs in places that are too dangerous to be accessed by the surgeon. Here, additional therapy options were needed.

The advances on the field of radiation by Becquerel, Roentgen and Curie laid the foundation for a second option of cancer treatment: radiation therapy.[19] The principle is that the cancer is exposed to high-energy

radiation (photons, protons and particle radiation) either from an external beam or from radioactive particles (seeds) implanted directly into the tumor.[20] The ionizing radiation induces DNA damage in cancer cells. Healthy cells are hit as well but are more efficient in repairing damage.[21] Cancer cells with defects in stability genes like BRCA1 struggle to repair DNA damage adequately, which results in cell death or mitotic arrest.[21,22] Radiation can be used as adjuvant or neoadjuvant therapy. Adjuvant means that after resection of the tumor the area is radiated to kill potential residual cells. Neoadjuvant therapy is used before surgery to shrink the tumor mass before resection.[20]

4.1.6.2 Chemotherapy

The term chemotherapy was coined by Paul Ehrlich at the end of the 19th century. Ehrlich originally used that word to describe the chemical treatment of a disease.[19] The first chemotherapies for cancer treatment were used in the 1940s. The basis of anti-cancer chemotherapy is to target rapidly proliferating cells. However, these drugs do not distinguish between cancer cells and other rapidly proliferating cells, i.e. intestinal epithelium, blood and immune cells.[23] Depending on their mechanism of action, chemotherapeutic drugs act cell-cycle specifically or non-specifically.[23] The following paragraphs exemplify the common classes of chemotherapeutics and their respective mechanism of action in a dividing cell (**Figure 3A**).

Alkylating reagents

Observations during the Second World War showed that soldiers exposed to mustard gas suffered from leukopenia, lack of leukocytes. Based on this observation, in 1943 pharmacologists used nitrogen mustard, a derivative of mustard gas, to treat lymphomas.[19] Following these first treatments, cyclophosphamide was synthesized as a nitrogen mustard derivative. Today, cyclophosphamide and cisplatin are still used in clinics. Alkylating agents add alkyl groups to both mitochondrial and nuclear DNA resulting in strand breaks or in point mutations from C:G to T:A base pairs. Accumulation of these DNA alterations results in programmed cell death. This drug acts cell-cycle unspecifically. Adverse effects of alkylating agents are male infertility due to reduced sperm production and the risk of secondary malignancies due to the mutagenic activity of DNA alkylation.[23]

Anti-metabolites

Anti-metabolites were first developed by Sidney Farber in the late 1940s. He treated children suffering from acute lymphoblastic leukemia (ALL) with the folate antagonists aminopterin and amethopterin (now: methotrexate) and achieved first remissions for this until then incurable disease.[19] Anti-metabolites are analogs of nucleic acids or their precursors, mimicking purines (6-Mercaptopurine), pyrimidines (5-Fluoruracil, Cytarabine), or folate (Methotrexate). During DNA replication in S-phase these analogs can be incorporated into the DNA or inhibit enzymes that are essential for nucleotide synthesis (Methotrexate: dihydrofolate reductase, 5-FU: thymidylate synthetase).[23]

Anthrayclines

Anthracyclines are molecules derived from actinobacteria. The most commonly used drugs of this class are doxorubicin and mitoxantrone. Anthracyclines have three mechanisms of action. One, they generate

free oxygen radicals that in turn damage DNA strands and membranes. Second, anthracyclines can intercalate into the DNA blocking DNA synthesis in S-phase. Lastly, these drugs can also inhibit topoisomerase II, preventing relaxation of supercoiled DNA. Through these effects anthracyclines block replication and transcription which induces cell death. Anthracyclines exert cardiac toxicity damaging the cardiac muscle.[23]

Vinca alkaloids

Vincristine and vinblastine are alkaloids extracted from the periwinkle plant and are M-phase specific drugs. Both drugs block tubulin assembly and thus interfere with the spindle apparatus during mitosis.[23]

Topoisomerase inhibitors

As the name suggests, these drugs block the supercoiling activity of topoisomerases I or II thus preventing DNA transcription and replication. Irinotecan and topotecan are type I inhibitors, etoposide and amsacrine (and doxorubicin) are type II inhibitors, targeting the S-G2 transition.[23]

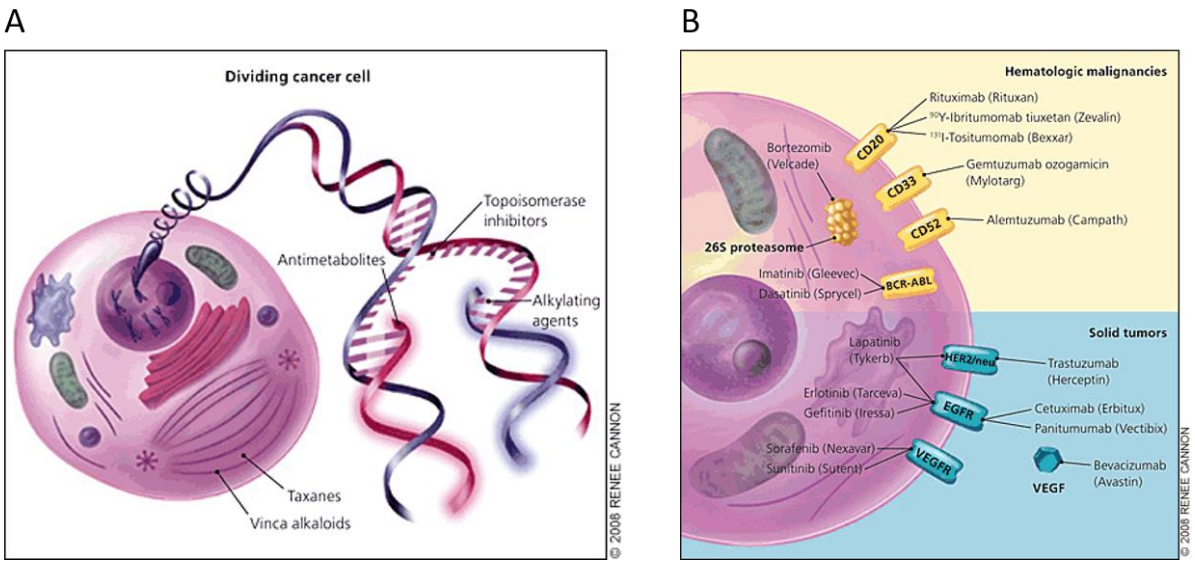

***Figure 3**: Targets of anti-cancer drugs: **A)** common targets of conventional chemotherapeutic drugs in a dividing cell. **B)** Cellular targets for precision medicine in hematological malignancies (shaded yellow) or solid tumors (shaded blue). Adapted from[24]*

4.1.7 Targeted therapies

Conventional therapy comprises the three options mentioned above and is still the standard of care treatment for most cancers. Their advance in the last century increased remission rates and improved survival of cancer patients. However, these therapy options come with some major drawbacks. Surgery does not affect distant (micro-)metastases and cannot be applied if tumors grow in unfavorable places. Radiation, while potent, also damages surrounding tissues and exposes healthy regions to dangerous doses of radiation. Chemotherapy does not target cancer cells specifically, and can have adverse effects in the whole body, especially on other rapidly dividing cells. While combination of these therapies helps to reduce the individual dose of each agent, it still cannot fully reduce severe adverse effects. Studies show that the rate of noncancer deaths in cancer patients is increased compared to the healthy population. In the case of chemotherapy, a study could show that around 8% of cancer-patient deaths

can be attributed to the therapy.[25–28] Due to these drawbacks of conventional therapy, the field of precision medicine is advancing and already being implemented into the standard of care regimens.

The goal here is, to find new selective treatment strategies directed against cancer-specific vulnerabilities while sparing healthy cells (**Figure 3B**). These vulnerabilities are reflected in the concept of oncogene addiction: In many cases, by massively favoring a single oncogenic pathway through mutation or gene amplification, cancer cells gain growth advantages but also become reliant on these signals. Interruption of this pathway would have detrimental effects on the cancer cell. Cancer genetics and genomics reveal which genes are mutated or aberrantly expressed in an individual cancer cell population, thus pinpointing the "Achilles heel" of these cells.[29] The sum of these cancer-specific pathways and features cumulates in the cancer cell obtaining the hallmarks of cancer, which are therefore sought to be targeted (**Figure 2**).[8]

A concept related to oncogene addiction is synthetic lethality. It describes the concept that of two genes, loss of either gene can be tolerated if the other gene can compensate. Loss of the second gene is detrimental for the cell. Synthetic lethality has been described in BRCA1-deficient tumors. The stability gene BRCA1 is important for the repair DNA double-strand breaks through homologous recombination (HR). BRCA1$^{-/-}$ cancers adapted to this loss and show genomic instability.[30,31] These cancers rely on other mechanisms to access this HR for DNA repair, in this case a protein called poly (ADP-ribose) polymerase (PARP-1). Thus, inhibition of PARP-1 is well tolerated in healthy cells, as these can still compensate loss of PARP-1 activity through BRCA1 (**Figure 4**).[31,32] BRCA1$^{-/-}$ cancer cells however, have now lost all their means to activate HR and as a result accumulate too much genomic damage to tolerate (**Figure 4**).[31] This overload in DNA damage leads to cell cycle arrest and programmed cell death (see below) in cancer cells.[30,32]

The next chapters exemplify ways to specifically target proteins in and on cancer cells.

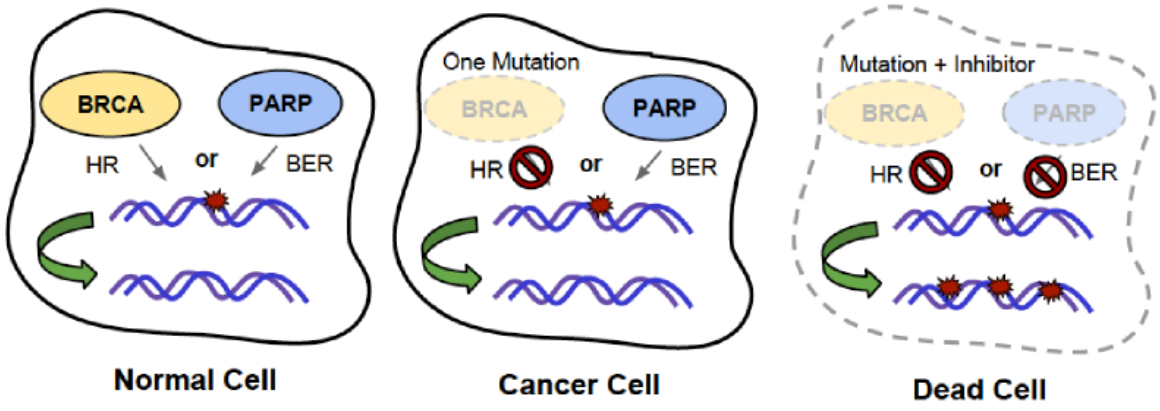

***Figure 4**: Model of synthetic lethality: Left: in a normal cell, BRCA and PARP can activate homologous recombination (HR) or base excision repair (BER) to repair DNA damage. Center: a cancer cell deficient for BRCA and thus for the HR pathway relies on PARP and BER to get rid of overwhelming DNA damage. Right: Inhibiting PARP rids the cancer of its last mechanism to contain DNA damage and prevent subsequent cell death. A healthy cell will still have the BRCA pathway to compensate for loss of PARP activity. Adapted from[33]*

4.1.7.1 Small molecule kinase inhibitors

Small molecules are defined by their low molecular weight of less than 900 Da, allowing them to diffuse through membranes to reach intracellular and even nuclear targets.[34,35] The majority of small molecule inhibitors target kinases. Kinases typically have a specific ATP-binding site and several adjacent pockets

whose composition differs between different kinases. Thus, the ATP-binding site and the pockets are targets for the design of specific competitive inhibitors.[36]

One of the most successful inhibitors is imatinib (Glivec® / Gleevec®) used to treat chronic myeloid leukemia (CML).[23] This inhibitor targets the pathognomonic fusion protein BCR-ABL, a tyrosine kinase acting as a strong oncogene (**Figure 3B**). Imatinib competitively binds to the ATP-binding pocket, disrupting the kinase potential. This treatment results in a complete response in 98% of the patients.[23,37,38] A second BCR-ABL inhibitor is dasatinib (Sprycel®). Dasatinib has a broader target spectrum and is used in imatinib-resistant CML.[23,39]

Other kinase inhibitors target the intracellular kinase domains of receptor tyrosine kinases like VEGFR (sorafenib, Nexavar®) or growth factor receptors like EGFR (gefitinib, Iressa®).[23] Several kinase inhibitors are currently in pre-clinical development and clinical trials, targeting kinases, like BRAF (Vemurafenib)[40], AKT (Afuresertib)[41], or Aurora kinase (Alisertib)[42,43].

The major drawback of all these inhibitors however is, that they are prone to giving rise to resistance in cancer cells, for instance due to mutations in the binding region.

4.1.7.2 Non-kinase inhibitors

Importantly, also non-kinase targeting inhibitors have been developed and made it into the clinics. A prime example here is the PARP-1 inhibitor olaparib (Lynparza®), which has been approved for the treatment of BRCA-deficient breast cancer to exploit the very concept of synthetic lethality explained above.[44]

One crucial problem to date is targeting of transcription factors. Transcription factors lack enzymatic activity but rather exert their function through DNA-protein or protein-protein interactions. Thus, on the one hand, interaction surfaces are too large for small molecules to cover let alone disrupt.[45] On the other hand, apart from the DNA-binding domain, transcription factors are known to be intrinsically disordered, making it near impossible to obtain a crystal structure needed for drug design.[46] The efforts to directly target a transcription factor are exemplified by the search for a drug targeting c-MYC which to date, after years of research, still remains unfruitful.[47,48]

4.1.8 Immunotherapy

While all of the therapies described above directly target the cancer by exogenous means, another therapeutic concept is to stimulate the body's own defenses, the immune system, to fight cancer.

The immune system has a major role in controlling cancer development, as reflected by the increased cancer risk in immunocompromised patients. The estimation is, that each cell encounters approximately 20.000 events of DNA damage each day.[49] Most of the damage is repaired by the cell-intrinsic DNA damage repair machinery and if not, the cells undergo programmed cell death (see below). Cells that acquire a mutation and continue to proliferate however, are in danger of transforming. Here, the immune system recognizes these damaged cells and eliminates them. Each cell of the body fragments part of its proteins and presents these antigens on its surface using the major histocompatibility complex I (MHC-I). Cells of the adaptive immune system, especially $CD8^+$ cytotoxic T-lymphocytes (CTL) scan the

cells for foreign antigens.[50] A mutated peptide presented on MHC-I is recognized by the T-cell receptor (TCR) as a foreign antigen, also called a neo-antigen or tumor-associated antigen (TAA). CTL activation needs to be bolstered by co-stimulatory signals present on an antigen-presenting cell (APC) of the immune system (e.g. dendritic cell) to reach sufficient activity. Co-stimulatory signals are mediated through engagement of CD28 on the CTL surface and B7 ligands (e.g. CD80 or CD86) on the APC. Sufficiently active CTLs eliminate the cancer cell by secretion of perforin, granzyme, and interferon-γ, as well as expression of FAS-ligand to induce extrinsic apoptosis (see below).[50]

However, as described by Hanahan and Weinberg in their Hallmarks of Cancer, evasion from immune surveillance is another characteristic of cancer cells.[8] Cancer cells can down-regulate MHC-I expression, express inhibitory T-cell ligands (e.g. programmed cell death protein ligand 1, PD-L1), or secrete immunosuppressive cytokines (e.g. TGF-β). All these mechanisms result in suppression of the anti-cancer function of immune cells in the cancer tissue. The following sections will briefly introduce new strategies of re-activating the immune response against cancer.

Monoclonal Antibodies / checkpoint inhibitors / BiTEs

Since the 1980s monoclonal antibodies (mAbs) have been designed to act against a large variety of antigens on the surface of or secreted by cancer cells. mAbs work on the one hand by blocking ligand binding to important receptors, e.g. growth factor receptors.[19] On the other hand, mAbs recruit cells of the immune system like lymphocytes and natural killer (NK) cells to the cancer cell to exert antibody-dependent cellular cytotoxicity (ADCC). The first mAb approved for cancer therapy was rituximab (Rituxan®), a mAb directed against CD20, for the treatment of non-Hodgkin lymphoma.[19] Other important mAbs, approved for cancer therapy are trastuzumab (Herceptin®) targeting EGF-R in breast cancer, or bevacizumab (Avastin®), targeting molecules of vascular endothelial growth factor (VEGF) released by cells of the cancer microenvironment.[19] Thus, bevacizumab prevents binding of VEGF to its receptor and blocks angiogenesis, another Hallmark of Cancer.[7]

The most recent breakthrough in immunotherapy relies on so-called immune checkpoint inhibitors. These mAbs bind and block immunosuppressive checkpoints, like PD-L1 on the cancer cells, PD1 on CTLs, or CTLA-4 on APCs that way reactivating CTL function.[51] Immune checkpoint inhibitors that have already been approved for the clinics in various cancer types are atezolizumab (anti-PD-L1), nivolumab, pembrolizumab (both anti-PD1), and ipilimumab (CTLA-4).[51]

Another antibody-based approach is the fusion of two scFv-fragments from mAbs to engage two different antigens, one on the cancer cells and the other CD3, on the T-cell surface. These so called, bi-specific T-cell engager (BiTE) work by bringing T-cells into close proximity of a cancer cell to exert their function. The first BiTE to be approved for the clinics is blinatumomab (Blincyto®), targeting CD19 on acute lymphoblastic leukemia (ALL) cells.[19]

Chimeric antigen-receptor (CAR) T-cells

CAR T-cells mark the most recent milestone in cancer therapy. In brief, the principle is based on adoptive T-cell transfer where patient T-cells are isolated, primed, and reinjected into the patient. In this specific setting, isolated T-cells are genetically modified to express a chimeric antigen-receptor.[52] The CAR consists of the antigen-binding region of a mAb engineered to the TCR and accessory signaling

molecules.[52] In this way, CAR T-cells can engage almost any cancer cell expressing the antigen without the need for MHC-based presentation. In 2017 the FDA approved the first CAR T-cell therapy tisagenlecleucel (Kymriah®), directed against CD19 in ALL.[53,54]

4.2 PEDIATRIC CANCER

4.2.1 Statistics adult vs. pediatric cancers

Pediatric cancers include those affecting patients under the age of 14 years but also adolescents up to the age of 19 years. By that definition, with approximately 175.000 new cases per year, pediatric cancer only comprises 1% of all cancer cases. Nevertheless, cancer is the most common disease-related cause of death in children and adolescents.[10]

Pediatric cancer differs from adult cancer in a variety of factors. The most apparent difference is the spectrum of cancer types. While the large majority of cancer types in adults are carcinomas (**Figure 5A**), these cancers almost never occur in children and adolescents.[55] Instead, hematological malignancies (e.g. ALL) and cancers of neuroectodermal origin (e.g. neuroblastoma) comprise the majority of pediatric cancers compared to the those of the adult population (**Figure 5B**).[55] Importantly, the spectrum of pediatric cancers differs also between children (0-14 year) and adolescents (14-19 years) where non-Hodgkin lymphomas are most prevalent. Moreover, compared to adult cancer the spectrum of pediatric cancers consists to a much larger proportion of sarcomas.[55]

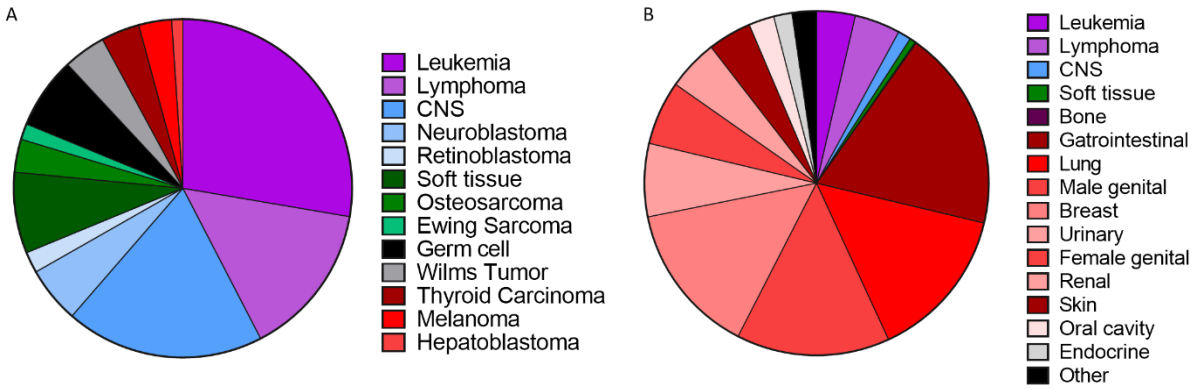

***Figure 5**: Cancer incidence in **A)** children (0-19 years) and **B)** adults. Purple: hematological origin, blue: CNS origin, green: sarcomas, red: epithelial origin (carcinoma). Adapted from[56,57]*

4.2.2 Etiology

Also in their etiology pediatric cancers appear profoundly different from adult cancers. In many cases the exact etiology of pediatric cancer is unclear. While adult cancers, especially carcinomas have years to accumulate mutations, pediatric malignancies understandably do not have this range of years. As a result, pediatric cancers in general harbor far fewer mutations than most adult carcinomas (**Figure 6A-B**).[5,55] While adult cancers, such as lung adenocarcinoma can harbor over 200 nonsynonymous mutations, pediatric cancers present on average with 9.6 mutations.[5] Another explanation for this low mutational burden is that children are not exposed to mutagenic environmental noxae, like tobacco

smoke, for as long as adults. Furthermore, in a recent study it was estimated that of over 1100 childhood cancer patients only 8% showed a hereditary predisposition.[58] In conclusion and with respect to the short latency of onset, it is therefore more likely that rather than environmental risk factors these few oncogenic aberrations already occur during early development and drive tumorigenesis. The onset of disease seems to depend on the differentiation stage of the cell of origin.[58]

Strikingly, pediatric cancers often show higher levels of chromosomal aberrations, such as translocations.[59] This high degree of chromosomal abnormality might indicate that single but drastic events of genomic rearrangement could lead to the development of driver aberrations. Indeed, one such event, named chromothripsis, has been found to take place in a variety of cancers and in up to 25% of all bone cancers. [60,61]. Chromothripsis describes a phenomenon of a single cellular crisis where multiple chromosomal breaks occur and 10-100 genomic rearrangements take place.[61] In medulloblastoma chromothripsis has been linked to p53-deficiency, likely as a requirement for cancer cells to survive the massive chromosomal damage.[62]

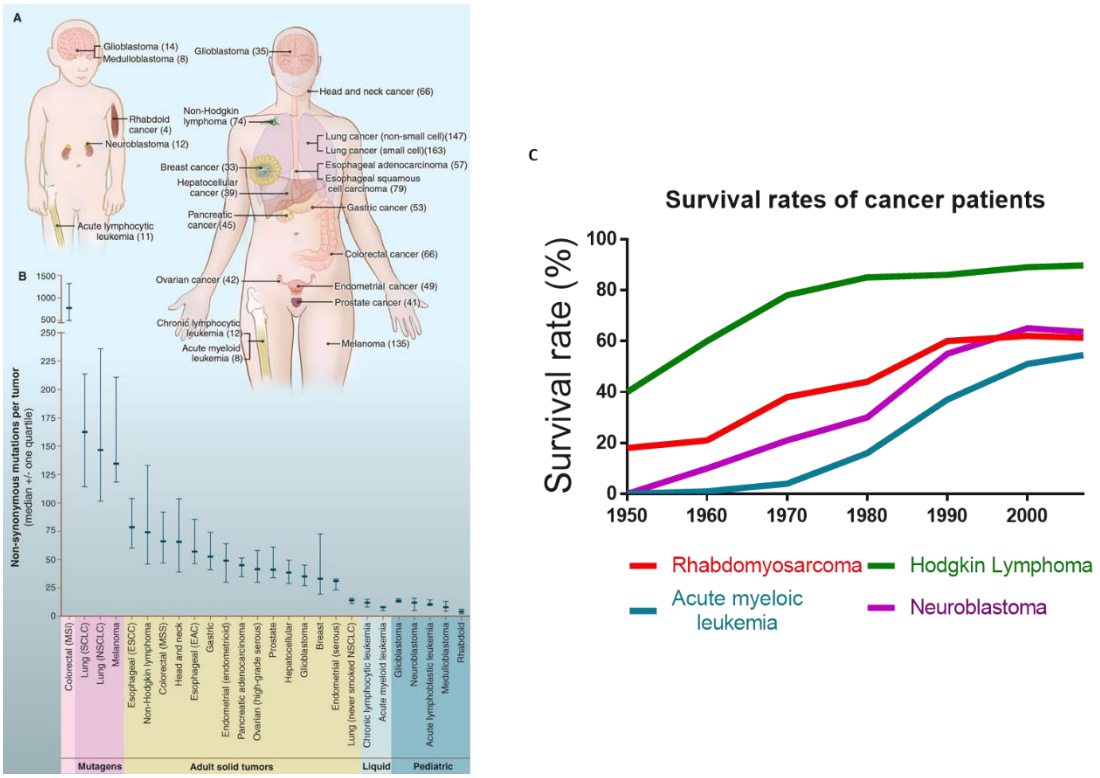

***Figure 6**: **A)** Mutational burden of cancers in children and adults depending on the site of origin. Numbers give the amount of non-synonymous mutations. **B)** Graph exemplifying the mutational burden of mutagen-induced cancers (pink), adult solid tumors (yellow) and pediatric cancers (blue). Adapted from[5] **C)** Increase of 5-year survival rates of four childhood cancers over the past decades. Adapted from[63]*

4.2.3 Prognosis & therapy

In spite of the lower mutational burden of pediatric cancers, their genetic heterogeneity is complex, which results in a large intertumoral and intratumoral heterogeneity. The intertumoral heterogeneity makes predictions of outcomes between patients very difficult, even though they might share histological characteristics. The intratumoral heterogeneity means that in some cases different subsets of the cancer of one patient may respond differently to therapy.[64]

Nevertheless, the development of advanced standard treatment protocols comprising surgery, radiotherapy and chemotherapy in the 1950s-1970s led to an overall increase in cure rates of pediatric

cancer of 80%. However, there is a high extent of heterogeneity in 5-year survival rates. Some cancers, like Wilm's tumor or Hodgkin lymphoma show 5-year survival rates over 90%, while metastasizing sarcomas still have a dismal prognosis of less than 30% survival after 5 years.[65] What is more, in these solid tumors the cure rate has stagnated for the last four decades (**Figure 6C**).[65]

Apart from the heterogenous response of pediatric cancers to conventional therapy, another major drawback of standard therapy is the adverse effects these aggressive regimens can impose on the developing body of young patients. Apart from the immediate adverse effects like nausea, hair loss and leukopenia, children suffer from defects in their cognitive development and potential infertility affecting their later life. Other adverse effects include cardiopulmonary defects, like lung fibrosis or ventricular dysfunction, or central nervous system deficiencies like hearing loss.[66]

Most importantly, due to the mutagenic potential of radiation and certain chemotherapeutics, e.g. alkylating agents and anthracyclines, surviving patients have a 19-fold increased risk to die from secondary malignancies compared to the healthy population.[66] These malignancies can occur years if not decades after treatment, and most commonly arise as breast, bone and thyroid cancers.[66]

In spite of the huge progress in the development of targeted therapies for adult cancer in the last decades, bringing these therapies into the clinics for pediatric cancer treatment has proven difficult. The reason why only so few targeted therapies can be tested for childhood malignancies is that dosing proves difficult, as children over the years of their development undergo several changes in a variety of influencing factors, such as activity of metabolizing enzymes, pH of the gastric environment, or the biodistribution of drugs.[66] Owing to these changes, drug dosing needs to be thoroughly calculated to avoid overexposing or undertreatment with the new drug.[66] Another issue is that due to the low mutational burden of pediatric cancers, many of the precision medicine targets of adult cancer are simply not applicable in pediatric malignancies.

This lack of neo-antigens is also the reason why immunotherapeutic approaches so far have had only modest effects. In recent years, only few immunotherapies emerged that made it to the clinics for pediatric cancer treatment. One such example is dinutuximab (Qarziba®), a chimeric mAb against disialoganglioside GD2, which has been approved in the EU for treatment of neuroblastoma.[67] Furthermore, the CAR T-cell therapy Kymriah® against CD19 has also been approved for childhood ALL.[54,68]

4.3 RHABDOMYOSARCOMA

As explained above, sarcomas are much more common in children and adolescents than they are in the adult population. Sarcomas are cancers in tissues with mesenchymal origin like bone, muscle and soft tissue.[9] Soft tissue sarcomas comprise approximately 7% of all pediatric cancer entities and of this group, rhabdomyosarcomas (RMS) make up 48% of all cases, thus being the most common soft tissue sarcoma (**Figure 5**).[55] Rhabdomyosarcoma belongs to the group of small, round blue cell tumors of childhood that also comprises Ewing's sarcoma, lymphoma, and neuroblastoma.[69]

Though mostly a sporadic disease, some studies could link hereditary diseases to the occurrence of RMS. These predisposing diseases are Li Fraumeni syndrome (characterized by a loss of p53)[70], Neurofibromatosis I[71], and the Costello syndrome[72]. In families with Li Fraumeni syndrome, rhabdomyosarcoma is the most frequent pediatric cancer.[73]

4.3.1 Subtypes and prognosis

RMS comprises several subtypes of tumors that received their names from showing characteristics of skeletal myogenesis.[56] 5-year overall survival of patients, irrespective of RMS subtype is estimated to be around 65% [74] but there is a big disparity in prognosis between the subtypes (see below).

According to the WHO, these subtypes are distinguished based on histology, with embryonal RMS (eRMS) and alveolar RMS (aRMS) being the most common ones (**Figure 7A**).[11] As the other subtypes, like pleomorphic or undifferentiated RMS are less common and mostly occur in the adult population,[56] they will not be focused on in the next paragraphs.

Approximately 60% of all RMS cases are accounted for by eRMS, which occurs most commonly in the head and neck region, as well as in the genitourinary tract. Its histology shows features of undifferentiated skeletal muscle. Incidence of eRMS is highest in children under the age of 5 years, with a second peak of incidence during adolescence.[75]

In contrast, aRMS tumors comprise about 20% of RMS cases and most commonly arise in the trunk and extremities. The defining histological features of this subtype are alveoli-like structures in the tissue, similar to those of the lung. Occurrence of this subtype is most common in adolescence.[75]

Risk stratification at diagnosis is based on the TNM-staging of malignant tumors as well as a system of several factors introduced by the Intergroup Rhabdomyosarcoma Study Group (IRSG). Most importantly, metastasis status has a huge impact on outcome of the patients.[76]

Of all RMS subtypes, eRMS has the most favorable prognosis with a 5-year survival rate of almost 70% if not metastasizing. aRMS tumors show a far worse prognosis with 5-year survival only around 34%.[77,75] Irrespective of their subtype, 5-year overall survival of metastasizing eRMS and aRMS cases drops to a mere 11% (**Figure 7B**).[78–80] Metastases most commonly occur in lung, lymph nodes and bones.[75] Overall, prognosis is worse for adults and adolescents, as well as infants, while children between 1-10 years have a better prognosis.[75]

4.3.2 Molecular characteristics

The two major RMS subtypes not only differ in terms of their histology and clinical prognosis but also their mutational landscapes. Generally, both subtypes have a low mutational burden, with only 0.31 mutations per mega base (Mb) in protein-coding regions.[84]

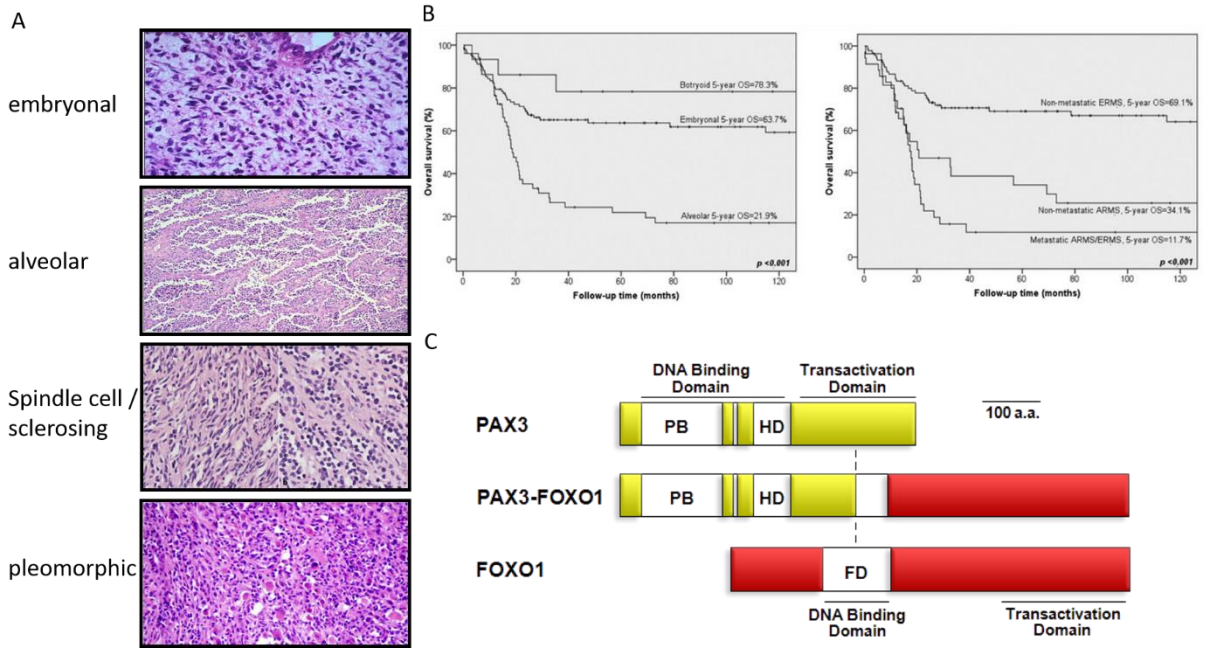

***Figure 7**: Rhabdomyosarcoma. **A)** Histologies of the four most common subtypes of RMS. Adapted from[81,82] **B)** Kaplan-Meier survival curves comparing three overall survival of the three given subtypes (left panel), or overall survival of non-metastatic vs metastatic diseases (right panel). Adapted from[77] **C)** Schematic of the fusion protein PAX3-FOXO1 and its respective wild-type components. Adapted from[83]*

In 80% of all aRMS cases, tumor cells harbor a reciprocal chromosomal translocation involving chromosomes 1 or 2 and another chromosome which leads to the fusion of one of the paired box genes PAX7 or PAX3 with another gene (**Figure 7C**) (see below).[80] With 0.1 protein-coding mutations per Mb, fusion-positive aRMS tumors harbor even fewer mutations than RMS tumors overall, accounting for only 6.4 somatic mutations per tumor, where only a median of 2.5 mutations are in expressed genes.[85] Interestingly, in sequencing studies, no recurrent single nucleotide mutations were found but instead genomic amplification of distinct regions was observed.[84,85] Most commonly, the regions 2p24 and 12q13-q14, which code for the oncogenes *MYCN* and *CDK4,* respectively, were amplified (**Figure 8**).[86] In contrast to neuroblastoma, amplification of these genomic loci in aRMS patients is not associated with worse outcome and overall survival.[87] As the PAX fusion proteins are oncogenic transcription factors that significantly influence aRMS biology, this subtype can be classified as a transcriptionally driven disease.

In contrast to aRMS, eRMS tumors harbor more single nucleotide mutations and display a larger amount of karyotype complexity and a heterologous histology.[84] Most often genes of the RAS pathway are mutated, for instance NRAS, KRAS or HRAS.[85] Furthermore, fibroblast growth factor receptor 4 (FGFR4) was found to be mutated in several cases, as well as phosphoinositide-3-kinase (PI3K) **(Figure 8**).[85] Moreover, among genomic alterations, most frequently loss of heterozygosity 11p15.5 was found which is also associated with other tumors, like Wilms tumor or hepatoblastoma.[84] Owing to these alterations, eRMS is considered a signaling driven disease.

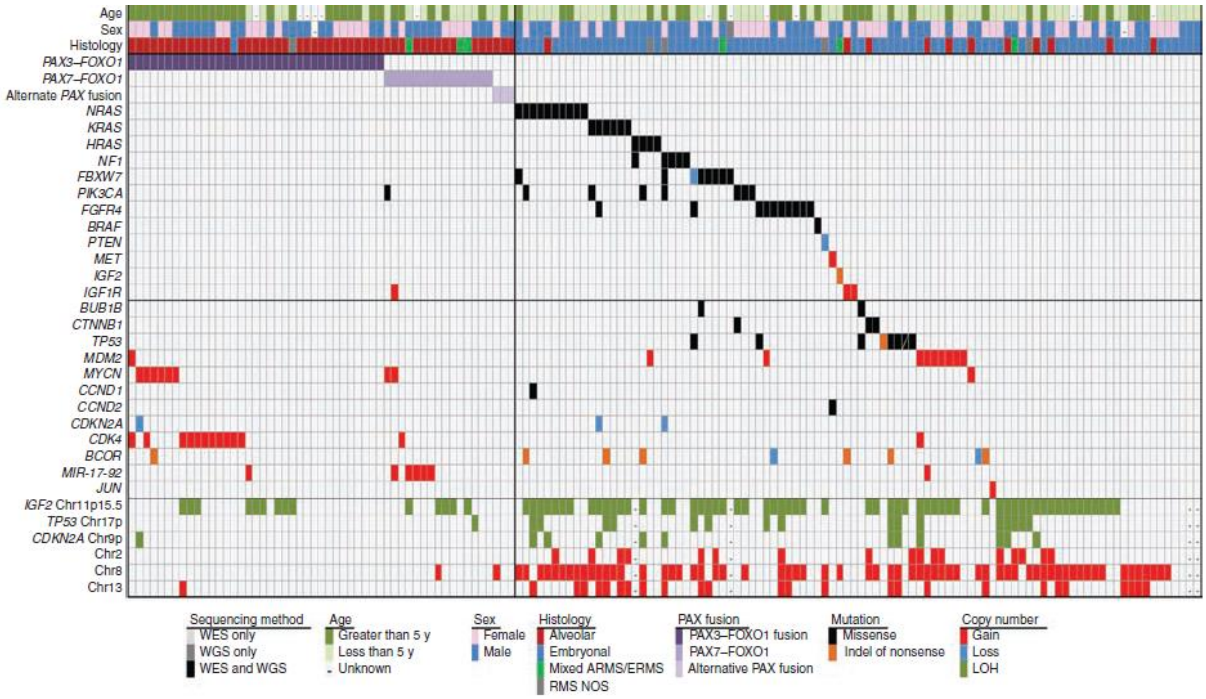

***Figure 8**: Mutational landscape of RMS tumors comparing alveolar RMS (left) and embryonal RMS (right). Each column represents one patient sample. Data based on whole-genome and/or whole-exome sequencing. Adapted from*[85]

4.3.3 PAX-FOXO1 fusion genes

The unique occurrence of the PAX fusion genes in most aRMS cases has drawn attention of researchers and made it a well-studied target. Indeed, the importance of these oncogenic transcription factors for aRMS tumor progression and maintenance could be demonstrated in several studies.

Through reciprocal translocation, in most cases, the N-terminal DNA-binding domain of a *PAX* gene is fused in frame to the transactivation domain of the forkhead box gene *FOXO1* located on chromosome 13.[88,89] In fewer cases, PAX genes are fused to NCOA1[90,91] or INO80D[85]. The most common fusion proteins are PAX3-FOXO1 and PAX7-FOXO1, expressed in 60% or 20% of all aRMS cases, respectively (**Figure 8**).[85,92,93]

Clinically, fusion-negative aRMS cases have a much more favorable prognosis similar to that of eRMS patients. Among fusion-positive cases, patients harboring the PAX3-FOXO1 rearrangement have a poorer prognosis than those with the PAX7-FOXO1 fusion, although their transcriptional program does not differ.[94]

PAX3-FOXO1 drives transcription of several hundreds of genes. Among these genes are markers of muscle differentiation, which reflects the natural function of PAX3 in differentiation processes.[95] Further genes activated by PAX3-FOXO1 are known oncogenic factors like FGFR2, FGFR4, MYCN and ALK.[96] These data indicate that, through its specific transcriptional program, the fusion has a major role in driving tumorigenesis of fusion-positive aRMS. Indeed, studies could show that ectopic overexpression of PAX3-FOXO1 in non-cancer cell lines and chicken embryonic fibroblasts is sufficient to drive transformation and anchorage-independent growth of these cells.[97,98] The role of PAX3-FOXO1 for tumor maintenance was demonstrated by anti-sense oligonucleotide-mediated knockdown of fusion gene expression. This study could show that loss of PAX3-FOXO1 expression resulted in cell death

underlining the essential role of the fusion protein for tumor survival.[99] *In vivo* studies demonstrated a loss of aggressive growth of aRMS cells after depletion of the fusion protein.[100]

PAX3-FOXO1 is post-translationally regulated through a large variety of modifications on sites also present in the respective domains of the wild-type proteins (**Figure 9A**).[95] In wild-type FOXO1 modification of these distinct sites regulates subcellular localization and protein stability.[101] One example of these regulatory modifications is acetylation of lysines 245 and 248 in wild-type FOXO1 by KAT2B.[102] This modification subsequently allows for phosphorylation of serines 256 and 319 by AKT, which in turn facilitates nuclear export and subsequent degradation of wild-type FOXO1.[103] Interestingly, while these sites also exist in PAX3-FOXO1, acetylation of the corresponding lysines K426 and K429 has been found to stabilize the fusion protein rather than lead to its degradation.[104]

4.3.4 Therapy

Standard of care therapy for RMS today is the result of numerous studies conducted by the IRSG (now Children's Oncology Group, COG) since 1972 and the European pediatric Soft tissue sarcoma study group (EpSSG). [105,106] In general, patients receive a conventional multimodal therapy consisting of surgery, radiation and chemotherapy. The exact regimen strongly depends on the individual risk stratification of the tumor. Assessment of risks has evolved over the past decades to optimize the treatment regimen for each individual patient.[76] Apart from TNM staging, the IRSG proposed and refined a clinical grouping staging system. This staging system also takes into account the unresectability of certain tumors sites, surgical status after surgical intervention, and metastatic status.[76] Based on this system, tumors are categorized into the four risk groups IRSG-I to IRSG-IV.

Surgery and radiotherapy are the localized therapies, where radiotherapy is recommended in sites where the tumor cannot be completely resected, or not resected at all.[76,105] Adjuvant radiotherapy was shown to improve outcome in aRMS patients also after complete resection of the primary tumor.[107]

However, due to drastic side effects of radiotherapy such as secondary malignancies or fibrosis, recommendations for the use of radiotherapy in RMS patients differ between COG and EpSSG. [105,108–110]

Chemotherapy is still the major systemic means of RMS therapy. Generally the regimen, which was implemented in 1974, consists of vincristine, actinomycin D and cyclophosphamide (VAC).[111] Since then, the regimen has not undergone substantial alterations but was refined in terms of dosage and time points based on risk stratification.[76] In Europe cyclophosphamide is often replaced by ifosfamide (IVA).[112] Moreover, these alkylating agents are omitted in case of low risk diseases.[105] In certain intermediate-risk or high-risk cases topoisomerase inhibitors such as doxorubicin, etoposide, or irinotecan, are included in the regimen.[105] The EpSSG tried to establish high-dose chemotherapy protocols to avoid the need for radiotherapy.[76]

Implementation of the VAC regimen has led to a drastic increase in patient survival since the 1970s.[113] However, despite the increase in cure rates of up to 90% in low-risk patients, overall the curve of improvement has flattened during the last decades reaching a plateau of an overall of around 70% of patients surviving 5 years after localized disease.[113] This trend is likely attributed to the fact that the core systemic regimen has not changed for over four decades now.[113] Moreover, disseminated disease still has a dismal prognosis, and 30% of RMS patients experience relapse of the cancer. In these cases,

treatment choices are sparse and often consist of increased doses of the first line chemotherapy, with meagre outcomes.[114] The severity of this situation is reflected in 5-year survival rates that drop down to a mere 17% with the median survival time after recurrence being as low as 0.8 years.[114]

These dire perspectives substantiate the need for new therapy options in RMS treatment. To date, no targeted therapy approaches have entered the clinics, but research is focused on finding new options to treat RMS.

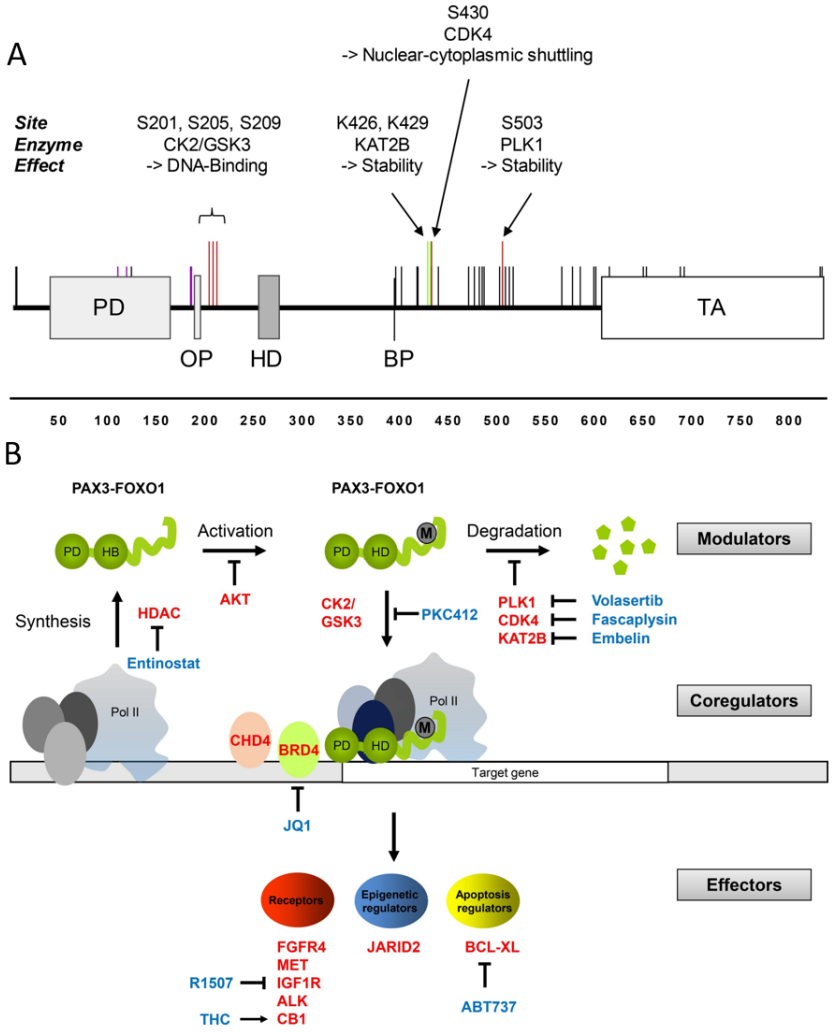

***Figure 9**: Targeting PAX3-FOXO1 biology. **A)** Schematic of the sites of post-translational modifications and their effects on the PAX3-FOXO1 protein. **B)** Possible levels to interfere with the "life-cycle" of the oncogenic transcription factor. Red: Target proteins, blue: targeted drugs available. Adapted from[95]*

4.3.5 Novel therapy approaches

For adult cancer, a variety of targeted therapy options exist. However, as mentioned before, transitioning from approved adult drugs to pediatric cancers proves cumbersome and at times impossible. Unfortunately, the same holds true for RMS treatment: Though various promising pre-clinical studies exist, in the clinic these drugs face many pitfalls, for instance intrinsic and acquired resistances. Due to these resistances, most pre-clinical and clinical studies investigate combination therapy effects rather

than monotherapy.[115] In case of aRMS, among the already low number of mutated genes (median of 2.5 in transcribed genes), even fewer are cancer-associated or druggable.[95]

Most of the recent studies use small molecules to target different receptor tyrosine kinases (RTK) and downstream signaling. Only a few examples are presented in the following paragraphs. One strategy relies on targeting insulin-like growth factor receptor 1 (IGFR-1) and anaplastic lymphoma kinase (ALK). Here, combination of ALK inhibition with the multi-kinase inhibitor sorafenib, or with the src inhibitor dasatinib showed synergistic effect in vitro.[116,117] Of note, many RMS tumors showed resistance to IGFR blockade. In some cases, enhanced activity of src or platelet-derived growth factor receptor beta (PDGFR-β) were attributed to this resistance.[118] Consequently, simultaneous inhibition of these proteins using the inhibitors pazopanib or crenolanib overcame resistance to IGFR blockade in vivo.[118] Resensitization to this IGFR-directed treatment could also be achieved using mTOR inhibitors or PI3K inhibitors.[119]

Other RTK-directed approaches target fibroblast growth factor receptor 4 (FGFR4), a target gene of PAX3-FOXO1, PDGFR, or EGFR.[120] Simultaneous treatment of VEGFR using sunitinib and the camptothecin analogue namitecan reduced RMS tumor growth in vivo.[121]

While most of the inhibitors, mentioned above, target RTKs many targeting approaches for different intracellular signaling cascades are being investigated as well. Targeting the PI3K/mTOR pathway showed a lot of potential *in vitro*, especially if combined with inhibition of the RAS/MEK/ERK pathway. [115,122]. Clinical trials however, combining the PI3K inhibitor buparlisip with the MEK inhibitor selumetinib, show that although anti-tumor efficacy can be increased this comes at the cost of increased toxicity, thus invalidating the idea of targeted therapy as an alternative to conventional therapy.[123] The RAS/MEK/ERK pathway is especially important in eRMS tumors, which often show mutations in this pathway. Among several approaches, one study found that inhibition of MEK acts synergistically with radiotherapy in vitro and potently diminishes the pool of cancer stem cells [124]. Combination of targeted therapy with conventional therapy is a much-pursued goal in pre-clinical research. Synergistic effects could be observed when combining inhibitors of the JAK/STAT pathway with doxorubicin or cisplatin in vitro.[115] Moreover, inhibiting GLI2 in the hedgehog pathway using GANT-61 in combination with mTOR-inhibitors or vincristine significantly reduced tumor growth in xenograft mouse models.[125,126]

Apart from small molecule inhibitors, immunotherapy-based approaches targeting RMS were heavily investigated in recent years, although, up until now, to no avail. The COG tried targeting the IGFR axis by using monoclonal antibodies (mAbs) against IGF1 in various pediatric sarcomas. However, phase II clinical trials showed no objective results on patient outcome.[127,128] Another phase I/II study investigates effects of combining a mAb against IGF1R with dasatinib (NCT03041701). As of July 2018, no results have been obtained. A phase II clinical trial by the EpSSG comparing a combination of the VEGFR-directed mAb bevacizumab with standard chemotherapy vs. chemotherapy alone showed no effect on event-free survival of patients[129] In another clinical trial by the COG, bevacizumab + standard chemotherapy (vinorelbine and cyclophosphamide) was compared to mTOR inhibition + standard chemotherapy. Here, the bevacizumab arm was discontinued due to significantly worse outcome compared to mTOR inhibition[130] A variety of other pre-clincal studies tried targeting different surface proteins on RMS tumors, like FGFR4, glypicans 3 and 5, or folate receptor 1, also with moderate success or none at all.[131]

Cell-based immunotherapy approaches are sparse for RMS treatment. Cho and colleagues could demonstrate that RMS cells are sensitive to activated NK-cell anti-tumor activity in vitro.[132] Another approach was to prime immune cells ex vivo using translocation-specific peptides found in different pediatric sarcomas, for example PAX3-FOXO1 in aRMS, and then re-introducing them into the patients to establish an immune response. The pilot study showed increased immune responses. However, only three aRMS patients were included which does not allow for firm conclusions.[133]

4.3.5.1 Targeting PAX3-FOXO1 biology

A unique opportunity for targeting aRMS arises from the expression of the oncogenic and crucial fusion protein PAX3-FOXO1, which is critical for maintaining tumor cell viability. Similar approaches targeting oncogenic fusion proteins, have great success in diseases like CML where the kinase inhibitors imatinib or dasatinib potently inhibit the BCR-ABL fusion. However, the case of aRMS, drug design is much more challenging owing to the fact that PAX3-FOXO1 is not a kinase but rather a transcription factor. As described above, designing small molecules to directly target a transcription factor is nearly impossible due to the lack of enzymatic activity (i.e. no ATP-binding pockets), large DNA-protein or protein-protein interaction surfaces, and the intrinsically disordered structure of the transactivation domain.[45] In order to take advantage of the important role of the fusion protein in aRMS, one needs to come up with indirect approaches interfering with PAX3-FOXO1 biology. **Figure 9B** shows possible strategies to indirectly target the transcription factor at different stages of its "life cycle".[95]

The first point with which to interfere is to reduce expression of PAX3-FOXO1 for example through epigenetic drugs. However, currently not much is known about the exact mechanisms regulating fusion gene expression. Still, studies could show that the histone deacetylase (HDAC) inhibitor entinostat could reduce PAX3-FOXO1 gene expression and aRMS tumor growth in vivo. [134] Of those HDAC inhibitors that are in clinical trials, panobinostat and vorinostat also showed anti-tumor effects against RMS in vivo. [135] Currently, a variety of HDAC inhibitors are in clinical trials for combination therapy of RMS with chemotherapeutic drugs. [95,136]

The second step to target PAX3-FOXO1 indirectly addresses its co-factors. Transcription factors work in concert with a large variety of epigenetic regulators and complexes to exert their function. Here, it could be shown that PAX3-FOXO1 is dependent on chromodomain helicase DNA-binding protein 4 (CHD4), a multi-domain protein that also has ATPase functions. Depletion of CHD4 was shown to have detrimental effects on aRMS viability similar to loss of PAX3-FOXO1.[137] However, currently no CHD4 inhibitor is available.[95] Another co-regulator, recently described, is the BET bromodomain protein BRD4.[138] Inhibition of BRD4 using the small molecule JQ-1 lead to loss of the PAX3-FOXO1 gene expression signature and showed anti-tumor effects in xenograft mouse models.[138,139]

The third and broadest step to interfere with PAX3-FOXO1 biology is to target its effectors, i.e. its target genes. Several of these target genes are known oncogenes and small molecules exist to target them. Among many others, these effectors and their inhibitors include RTKs such as IGF1R, FGFR4, met or ALK. [140–143]. Interestingly, also genes involved in apoptosis belong to the target genes of PAX3-FOXO1. For instance, BCL-X_L was found to be a target gene.[144] Indeed, RMS cells were shown to be susceptible to inhibition of BCL-X_L in combination therapy.[145] Conversely, PAX3-FOXO1 has also been implicated in higher expression of NOXA, thus priming RMS cells for cell death and making them sensitive towards

treatment with BH3-mimetics (see below). [146,147] However, a direct transcriptional upregulation of NOXA by the fusion protein has not been established so far.

Lastly PAX3-FOXO1 biology can be targeted through modulators of the fusion protein. Phosphorylation of the fusion protein by CDK4 has been shown to help localize the protein to the nucleus, thus enhancing its activity. Consequently, inhibition of CDK4 was shown to reduce transcriptional activity of PAX3-FOXO1 and delay aRMS xenograft growth in mice.[148,149] Furthermore, PAX3-FOXO1 stability is regulated through proteasomal degradation of the fusion protein.[95,150] It could be demonstrated that acetylation of PAX3-FOXO1 at K426/K429 by the acetyltransferase KAT2B (P/CAF) stabilizes the fusion protein.[104] Another important interactor of PAX3-FOXO1 is polo-like kinase 1 (PLK1), which has been shown to phosphorylate the fusion protein at S503 and thereby stabilize it.[150] Indeed, inhibition of PLK1 by volasertib reduced cell viability and diminished tumor growth in vivo.[150]

Although a large variety of targeting options exist, especially for PAX3-FOXO1 biology in aRMS, so far none of these approaches made it past clinical trials and the search for successful a drug combination is still a paramount goal in RMS research.

4.4 CELL DEATH

4.4.1 General

Cell death belongs to the life cycle of every cell, though how exactly a cell dies varies and depends a lot on the circumstances. Historically, scientists distinguished between only two distinct types of cell death: necrosis and apoptosis. Here, necrosis described an instantaneous and irreversible process as a result of the cell being exposed to injury or other external stresses, disrupting the integrity of its cell membrane. Apoptosis, on the other hand was described as an intrinsically regulated process, requiring active protein synthesis.[151,152]

In recent years however, scientists discovered a variety of distinct modes of cell death of which the majority are regulated and not instantaneous. A more fitting classification of cell deaths therefore is to distinguish between accidental cell death (ACD) and regulated cell death (RCD). While ACD essentially matches with the historical description of necrosis, apoptosis is only one variation of many forms of RCD.[153]

ACD can occur through physical stress (changes in pressure, temperature, osmotic pressure), chemical stress (pH variation), or mechanical (shear force).[153] As a result of a ruptured cell membrane, cytosolic and nuclear components of the dead cell are now exposed to the environment and thus serve as danger signals, so called danger-associated molecular patterns (DAMPs) for surrounding cells and the immune system.[153–155]

Opposed to ACD, cells have developed various genetically encoded mechanisms to induce RCD as a response to different intrinsic or extrinsic stresses to allow for the most adequate response. In the past years, more and more distinct modes of RCD have been characterized and will be in future research which is why since 2005, the Nomenclature Committee on Cell Death (NCCD) meets on a regular basis to define major cell death modalities on a morphological, molecular and genetic basis.[156] Knowledge of

these defined and regulated modes of cell death allows for new strategies of pharmacological intervention in various diseases, especially in cancer.
In the next paragraphs a few types of RCD will be described in more detail.

4.4.2 Apoptosis

4.4.2.1 Characteristics

The term apoptosis was first introduced in 1972 by Kerr and colleagues to describe a regulated or programmed form of cell death that is important for cell turnover in healthy tissues and for elimination of cells in embryonic development.[157] The name apoptosis (*ἀπόπτωσις*) is derived from the Greek word describing the falling of leaves from a tree.[157]

Morphologically, apoptotic cell death is characterized by nuclear condensation (pyknosis) and chromatin fragmentation while maintaining an intact cell membrane that herniates into smaller compartments called apoptotic bodies (blebbing).[9,157] The effect of keeping the cell membrane intact is that no DAMPs are released during apoptosis making this an overall immunogenically silent cell death, although there are exceptions as demonstrated recently.[158,159] Apoptotic bodies release and present "find-me" and "eat-me" signals to attract macrophages and activate phagocytosis, respectively. That way apoptotic bodies are cleared before spilling intracellular DAMPs into the extracellular space. "Find-me" signals, released from dying cells, are for example ATP[160,161] or sphingosine-1-phosphate (S1P)[162]. Triggering phagocytosis is achieved by presentation of phosphatidyl-serine (PS) on the outer leaflet of the lipid bilayer forming the cell membrane. Under normal conditions, PS is distributed asymmetrically only on the inner leaflet of the cell membrane. With apoptosis occurring, enzymes named scramblases are activated to shuffle PS to the outer leaflet.[163] There, PS is recognized by annexin receptors on the macrophages, triggering phagocytosis.[164] Failure of macrophages to clear apoptotic bodies results in a process called secondary necrosis and subsequent inflammation due to the release of DAMPs from failing membranes of former apoptotic bodies.[165]

Apoptosis is an energy-dependent form of cell death and is characterized by the activity of a proteolytic cascade of cysteine-aspartate specific proteases, caspases. These endoproteases have a wide variety of targets and are synthesized in inactive dimers.[166] Caspases are activated through proteolysis by other caspases, which is why one distinguishes initiator caspases and executioner caspases.[166] The executioner caspases 3, 6, and 7 are the convergence of all upstream caspases and cleave several hundreds of targets, including other pro-caspases. In this way, the executioner caspases drive the apoptotic processes and morphological changes:
Cleavage of lamins on the nuclear membrane is one factor thought to drive pyknosis. Inactivation of inhibitor of caspase-activated DNase (ICAD) releases the respective DNase responsible for DNA fragmentation.[167] Cleavage of poly (ADP-ribose) polymerase-1 (PARP-1) ensures that energy storages of the cell are not consumed due to DNA damage repair activity of PARP-1.[168] Cleavage of cytoskeletal proteins, like actin, vimentin and others, results in blebbing of the cell membrane and the formation of

apoptotic bodies.[9,169,170] Lastly, caspase-3 has been found to proteolytically activate Xkr8, the scramblase responsible for flipping PS on the outer leaflet.[171,172]

The executioner caspases can be activated by different initiator caspases depending on the upstream cue initiating the activation cascade. These cues can be intrinsic or extrinsic stimuli, which is why the following two major forms of apoptosis are distinguished.

4.4.2.2 Intrinsic apoptosis

Overview

Intrinsic apoptosis is also called mitochondrial apoptosis, as the signaling program revolves around this organelle. It is the most common mode of RCD activated by cells in response to several different perturbations comprising growth factor withdrawal, endoplasmic reticulum (ER) stress, overload with reactive oxygen species (ROS), mitotic defects, and, importantly, DNA damage.[173–175] The pivotal step of intrinsic apoptosis is the irreversible mitochondrial outer membrane permeabilization (MOMP) resulting in the release of mitochondrial factors triggering formation of a cytosolic caspase-activating protein platform that initiates the caspase cascade.[153]

Interactions at the outer mitochondrial membrane

MOMP is the result of an interplay between pro-apoptotic and anti-apoptotic members of the B-cell lymphoma 2 (BCL2) family, facilitating or antagonizing the formation of pores in the outer mitochondrial membrane (OMM), respectively. All members of this family share up to four BCL2-homology domains (BH1 to BH4).[176–178] The pores are formed by BCL2-associated X apoptosis regulator (BAX) and BCL2 antagonist/killer1 (BAK).[179] While BAX has been found to cycle between OMM and cytosol, BAK stays at the OMM. In response to the stress signals mentioned above, BAX and BAK are activated transcriptionally or post-translationally at the OMM, either directly or indirectly by pro-apoptotic BH3-only proteins.[153] BAX, for example, ceases retro-translocation to the cytosol and accumulates at the OMM upon apoptosis induction.[153,180]

Important members of the BH3-only proteins are p53-upregulated modulator of apoptosis (PUMA), BCL2-interacting mediator of cell death (BIM) and phorbol-12-myristate-acetate induced protein 1 (PMAIP1, NOXA).[181,182] These BH3-only proteins are upregulated transcriptionally upon cellular stress, for example in response to DNA damage that cannot be repaired.[153] Pro-apoptotic BH3-only proteins can be sub-grouped into activators and sensitizers. They home to the OMM where activators, like PUMA, BIM, and NOXA interact transiently with BAK and BAX to activate them through conformational changes.[153] BAK and BAX activation allows for the formation of homodimeric and heterodimeric pores resulting in MOMP.[183–186] Formation of dimers results in release of BH3-only proteins and subsequent dimer-by-dimer oligomerization increasing membrane permeabilization through a toroidal pore.[187]

The process of BAX/BAK activation is antagonized by the subfamily of anti-apoptotic BCL2 proteins comprising BCL2, BCL2 like 1 (BCL-X_L), BCL2 like 2 (BCL-W), BCL2 related protein A1 (BCL2-A1, BFL-1), and MCL1.[153] These anti-apoptotic BCL2 family members reside in the OMM and exert their function either by sequestrating BAX and BAK, preventing their oligomerization, or by sequestration of activating

BH3-only proteins.[188–191] Some anti-apoptotic BCL2 proteins (e.g. BCL-X_L) also function by promoting retrotranslocation of BAX from the OMM to the cytosol, thus depleting the pool of mitochondrial BAX.[192] Sensitizing BH3-only proteins, like BCL2 associated agonist of cell death (BAD), BCL2 modifying factor (BMF), or hara-kiri, BCL2 interacting protein (HRK), do not physically interact with BAX and BAK to activate dimerization, but rather promote MOMP by binding and sequestering anti-apoptotic BCL2 family members. This sequestration restricts anti-apoptotic BCL2 protein function.[193] However, the distinction between activator and sensitizer BH3-only proteins seems to be less stringent than previously estimated.[194–196] This is reflected in the binding preferences of BH3-only proteins to anti-apoptotic BCL2 family members. BID, BIM and PUMA can bind all proteins of that family, NOXA preferentially inhibits MCL1, and HRK inhibits BCL-X_L.[194] In certain situations, alternative interacting pairs have been described. Some data hint at possible interactions of BCL-X_L with NOXA.[197] Nonetheless, overexpression of sensitizer proteins in absence of activator proteins only induces minimal apoptosis. These results suggest that activator BH3-only proteins act downstream of sensitizers.[153,198]

Recent evidence also shows that BAX and BAK can self-activate, albeit with much slower kinetics, even in absence of pro-apoptotic BH3-only proteins and anti-apoptotic BCL2 family members.[198,199] Another non-canonical mechanism of BAX/BAK activation is that p53 not only acts as a transcriptional activator of pro-apoptotic proteins but also exerts its function by directly activating the pore forming proteins at the OMM.[200–202]

Apoptotic mechanisms after MOMP

Ultimately, MOMP leads to the release of contents from the mitochondrial intermembrane space, allowing movement of proteins such as cytochrome c somatic (CYCS) and second mitochondrial activator of caspases (SMAC) to the cytosol. Cytosolic CYCS is bound by apoptotic peptidase activating factor 1 (APAF1) creating a supramolecular protein platform called the apoptosome.[203] The apoptosome can bind pro-caspase 9 through its caspase recruitment domain (CARD).[203,204] Proximity of pro-caspases in the multimeric coordination of the apoptosome induces autocatalytic maturation of caspase 9 (CASP9)[204] which can catalyze the proteolytic activation of CASP3 and CASP7[205]. Caspase activity can be inhibited by X-linked inhibitor of apoptosis (XIAP). Cytosolic SMAC associates with XIAP to reduce its activity, thus precipitating apoptosis by allowing caspases to exert their function.[206,207] Other IAPs exert their function by upregulation of anti-apoptotic factors like CASP8 and FADD like apoptosis regulator (c-FLIP), or by ubiquitylation of caspases or the receptor interacting serine/threonine kinase 1 (RIPK1) triggering pro-survival NF-κB signaling.[153]

In spite of these anti-apoptotic mechanisms, evidence suggest that activation of executioner caspases marks a point-of-no-return in the apoptotic cascade.[208] Under physiological conditions there is an equilibrium of pro-apoptotic and anti-apoptotic molecules at the OMM which is sufficient to keep the cells from reaching this point-of-no-return. Due to increased metabolism and high levels of replicative stress, cancer cells also express higher levels of pro-apoptotic proteins.[209] To compensate this shift in the apoptotic equilibrium, cancer cells need to upregulate anti-apoptotic BCL2 family proteins and are highly dependent on their activity.[209]

A specific variant of intrinsic apoptosis is called anoikis, triggered by the loss of integrin-dependent extracellular matrix attachment.[210] In this way, anoikis prevents anchorage-independent growth and as

such is considered oncosuppressive.[210] Cancer cells need to become insensitive to this mode of cell death when losing contact inhibition and gaining metastatic potential.[210]

4.4.2.3 Extrinsic apoptosis

In contrast to intrinsic apoptosis, extrinsic apoptosis is induced by activation of membrane-bound receptors. The most common of apoptosis-promoting receptors are death receptors including FAS (CD95, APO-1), TNF-receptor 1 (TNFR1), TRAILR-1 and TRAILR-2.[153] Generally, ligand-mediated activation of death receptors leads to multimerization and the cytosolic assembly of a multiprotein complex called death-inducing signaling complex (DISC). DISC recruits CASP8 (and sometimes CASP10).[153,211] FAS and TRAILRs exist as pre-formed homotrimers stabilized by ligand binding which induces conformational changes in the intracellular death domain (DD). These changes allow for association of *Fas associated via death domain* (FADD) to the DISC.[212–214] FADD recruits CASP8 and facilitates the formation of a linear filament consisting of several CASP8 proteins. The proximity of CASP8 leads to homodimerization and autoproteolytic activation.[215]

TNFR1 activation results in the association of TNFR-associated death domain (TRADD) which recruits a complex consisting of TNFR associated factor 2 (TRAF2), TRAF5, c-IAP1, c-IAP2, and RIPK1.[153] Importantly, here receptor ligation does not necessarily result in cell death, but depends highly on the degree of receptor oligomerization and the post-translational modification of RIPK1. Deubiquitylation of RIPK1 favors its association with FADD and CASP8, while polyubiquitylation by IAPs results in pro-survival signaling.[153]

Two major pathways downstream of CASP8 activation have been described. In Type I cells (thymocytes and mature lymphocytes), CASP8 proteolytically activates the executioner caspases 3 and 7, which is sufficient for apoptosis.[216] Type II cells (e.g. pancreatic β cells, hepatocytes and most cancer cells) rely on XIAP to hamper CASP 3 and 7 activity. In these cases, CASP8 proteolytically activates BID. Cleavage of BID results in a truncated form (tBID) which localizes to the OMM where it acts an activator BH3-only protein to activate BAX/BAK-dependent MOMP and CASP9 activation.[216,217]

4.4.3 Necrosis / Necroptosis

Historically, the only distinction between cell deaths was apoptosis versus necrosis. Apoptosis was considered as genetically regulated cell death and necrosis was considered as ACD.[157] The necrotic morphotype is characterized by swelling and rupturing of the plasma membrane and spillage of the cytosolic contents into the surrounding tissue. However, in 1988 it could be demonstrated that treatment with TNF results in either apoptosis or necrosis depending on the cellular background.[218] Later research showed that indeed necrosis could be inhibited chemically and by genetic means, indicating that necrosis also is genetically regulated.[219] In analogy to apoptosis as RCD but considering its morphotype, this mode of cell death was called necroptosis.[153]

Similar to extrinsic apoptosis, necroptosis is also initiated by death receptors like FAS and TFNR1. As mentioned above, the complex recruited to the receptor contains TRADD and RIPK1. Deubiquitylation of RIPK1 results in dissociation from the receptor to form complex II including TRADD, FADD, RIPK1.[220] Here, CASP8 is recruited and its activity is crucial for the decision between apoptosis and necroptosis.

As described above, active CASP8 induces the apoptotic cascade. However, if CASP8 is inactive or absent from complex II, RIPK3 and mixed lineage kinase domain like pseudokinase (MLKL) are recruited to the complex which is then called necrosome.[220] RIPK3 phosphorylates MLKL, which induces oligomerization and translocation of these oligomers to the plasma membrane where they trigger membrane permeabilization.[221]

The necrosome can be inhibited chemically by the compound necrostatin-1 (nec-1) inhibiting RIP kinases,[222] but also intrinsically, for example by Aurora kinase A interaction with RIPK1 and RIPK3.[223]

4.4.4 Other modes of regulated cell death

In recent years a variety of distinct modes of RCD have been described with their characteristic features. Pyroptosis is an RCD linked to the innate immune responses. It features formation of an inflammasome in response to pattern recognition receptor (PRR) activation by pathogens or DAMPs.[224] Assembled Inflammasomes recruit and activate CASP1, resulting in maturation of pro-inflammatory interleukins (IL-1β, IL-18) and their release through permeabilization of the plasma membrane.[225]

Ferroptosis only recently emerged as a mode of cell death that does not rely on caspases or necrosome components.[153] It is induced in response to severe lipid peroxidation as a result of high levels of ROS and iron availability.[226]

Parthanatos occurs due to hyperactivation of the DNA damage response machinery, specifically PARP1, in response to severe alkylating DNA damage but also oxidative stress, hypoxia, or inflammatory signals.[227] PARP1 hyperactivation leads to rapid depletion of intracellular NAD^+ and ATP levels, resulting in a bioenergetic and redox collapse, as well as mitochondrial membrane depolarization followed by MOMP.[227]

4.4.5 Cell cycle control

Each cell can control progression of one phase to the next in order to avoid severe consequences of progressing in spite of existing damage. These instances control are called checkpoints. Cell cycle checkpoints are mechanisms to halt cell cycle progression upon DNA damage. The three major checkpoints are during late G1-phase, late G2-phase, and in mitosis (M-phase) (**Figure 10**). The G1 checkpoint controls the size of the cell, whether sufficient nutrients or growth factors are present, and whether the DNA is damaged and then allows for progression into the S-phase or subjects the cell into G0-phase, a quiescent non-proliferative state.[228] The G2 (also G2/M) checkpoint assesses DNA damage and replication errors after the S-phase and prevents transition into the M-phase unless damage has been repaired.[228] Lastly, the M checkpoint makes sure that all spindles are properly attached to the kinetochores to prevent errors in chromosomal segregation.[228] Both, the G2 and M checkpoints will be described in more detail below (*chapter 4.5*).

Should errors persist in spite of cell cycle arrest, cells can initiate cellular senescence or apoptosis. Even though cellular senescence and mitotic arrest involve the machinery for certain forms of RCD, they cannot be considered as cell death.[153]

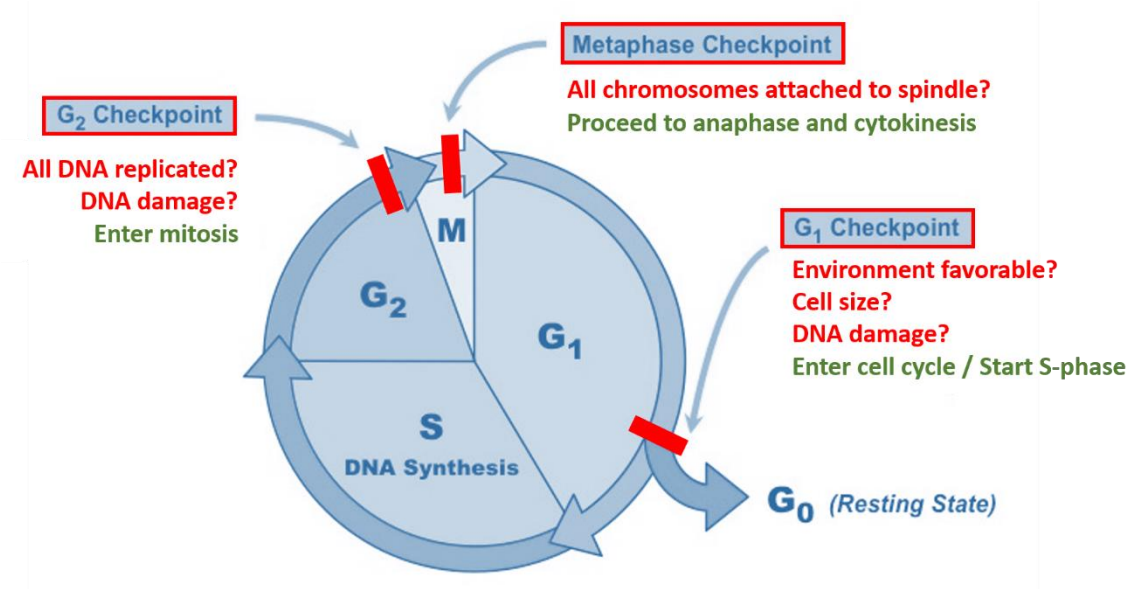

Figure 10: *Cell cycle phases and the major checkpoints. Checkpoints are indicated by red bars and the descriptions show what cues are assessed by each checkpoint (red) and what next step is initiated (green). Adapted from*[228]

4.4.5.1 Cellular senescence

Cellular senescence is a process in which cells permanently stop proliferating while still maintaining metabolic activity. In contrast to the quiescent state of G0-phase, senescent cells cannot be reactivated, for instance by mitogenic factors.[228] This process is thought to be involved in aging and the etiology of some age-related diseases. While cellular senescence is thought to be onco-suppressive, evidence hints at adverse effects in certain chemotherapeutic regimens and at recurrence of specific neoplasms.[229]

Senescence occurs in response to a variety of stimuli (see below) and is characterized by the inhibition of cyclin-dependent kinases (CDKs), absence of proliferation markers like Ki-67, and activation of the DNA damage repair (DDR) machinery.[229]

Replicative senescence

Replicative senescence is induced by telomere erosion due to high number of cell divisions. This process triggers the DDR mediated by ATR, ATM, CHK1, and CHK2, which in turn activate p53. As a result, cyclin-dependent kinase inhibitor 1A (CDKN1A, also called p21) is expressed to inhibit CDK2. Furthermore, the CDKN2A locus is de-repressed, which leads to expression of the tumor suppressors p16 and ARF.[153]

Oncogene-induced senescence

This process can function as another delay in early tumorigenesis, as high activity of certain oncogenes results in CDKN2A de-repression.[229] Moreover, excessive DNA replication and the concomitant errors result in DDR activation which in turn leads to p53 activation. Increased proliferation and resulting ROS can induce p38 MAPK resulting in increased transcriptional activity of p53.[153]

4.4.5.2 Mitotic catastrophe

Mitotic catastrophe is a specific, regulated mechanism that halts proliferation of cells unable to complete mitosis, for instance due to severe DNA damage and mitotic checkpoint failure (see below). As such it

can also be considered an important onco-suppressive mechanism. Mitotic defects can occur due to exogenous and endogenous cues.[230] Exogenous cues are drugs such as chemotherapeutics altering DNA replication, cell cycle checkpoints, microtubular dynamics, and chromosomal segregation. Endogenous sources can be high replicative or mitotic stress due to altered ploidy or due to deregulated factors important for DNA replication. While the exact mechanisms of how mitotic alterations are sensed are unclear, presumably they require p53 signaling. This hypothesis is supported by data showing that cells lacking p53 undergo a necrotic variant of RCD upon encountering mitotic defects.[231]

The fate of cells that are confronted with mitotic catastrophe seems to depend on the time spent in mitotic arrest. Typically, cells that arrest in mitosis for prolonged time undergo intrinsic apoptosis. Cells that escape mitotic arrest and enter interphase can undergo apoptosis later during the cell cycle or can enter senescence. Apart from apoptosis, mitotic catastrophe and subsequent senescence are the major effects from radiation therapy.[232]

Cancer cells require the ability to abrogate mitotic catastrophe to avoid complications when generating polyploid and aneuploid cells.

4.4.6 Therapeutic potential of cell death

Evasion from cell death is one of the original hallmarks of cancer proposed by Hanahan and Weinberg in 2000.[7] The strategies cancer cells come up with to evade cell death are diverse and offer valuable advantages in cancer progression. This evasion of cell death not only influences tumorigenesis but also the response of cancer cells to the various therapies available. Thus, knowledge of the specific strategies to evade cell death and the possible vulnerabilities can provide important new treatment options in cancer therapy.

4.4.6.1 BH3-mimetics

Cancer cells that regularly face replicative stress due to increased proliferation need to overcome the challenge of RCD.[209] One mechanism to protect themselves is that cancer cells upregulate anti-apoptotic BCL2 proteins that sequester stress-induced pro-apoptotic BH3-only proteins, and thus dramatically raise the threshold for these pro-apoptotic cues to induce MOMP (**Figure 11A**).[209] Thus, these cells not only show resistance against intrinsic stress signals but also against drugs inducing apoptosis. However, a major mechanism of action on which many chemotherapy regimens rely, is the induction of mitotic catastrophe and subsequent intrinsic apoptosis.[153] Therapy resistance, especially in relapsed samples, thus is likely explained by the insensitivity of cancer cells to apoptotic cues.[154,233–235] Fortunately, also here the concept of oncogene addiction applies. In this example, cancer cells already show a much higher apoptotic potential, they are primed for apoptosis, and rely solely on the anti-apoptotic function of certain BCL2 family members to prevent MOMP and subsequent caspase activation (**Figure 11B**).[209] A new idea for a therapeutic approach therefore has been to inhibit BCL2 proteins. The first effort in this direction was Genasense (oblimersen sodium), an antisense oligonucleotide directed against BCL2 in the treatment of chronic lymphocytic leukemia (CLL).[236] However, this drug showed only modest clinical activity.[237] Later, peptides and small molecules were designed, so called BH3-mimetics.[238,239] As the name suggests, their structures mimic the BH3-domain

and they insert into the hydrophobic grove of anti-apoptotic BCL2-family proteins, competing with their binding to BH3-only proteins and thus releasing the pro-apoptotic proteins.[238,240] BH3-mimetics were designed to target specific members of the anti-apoptotic BCL2 family. ABT-737 and its orally available derivative ABT-263 (Navitoclax) mimic the BH3 domain of BAD and thus target BCL-X_L with high affinity, but also BCL-2 and BCL-W.[241] Both compounds show strong pro-apoptotic effects in various preclinical models and in phase I and II clinical trials. However, they do not target A1 and MCL-1, and only showed these strong effects if MCL-1 was only weakly or not at all expressed expressed.[242–244] ABT-199 (Venetoclax, Venclexta®) is another derivative of ABT-737 but specifically targets BCL-2.[245] In 2016, ABT-199 was granted accelerated approval as a breakthrough therapy by the FDA for the treatment of patients with CLL harboring a 17p deletion.[246–248] In June 2018 approval was extended to second line treatment of all patients with CLL, irrespective of 17p status.[249,250] Further BH3-mimetics targeting different BCL2 proteins are currently under development, for instance the MCL-1 inhibitor S63845.[251]

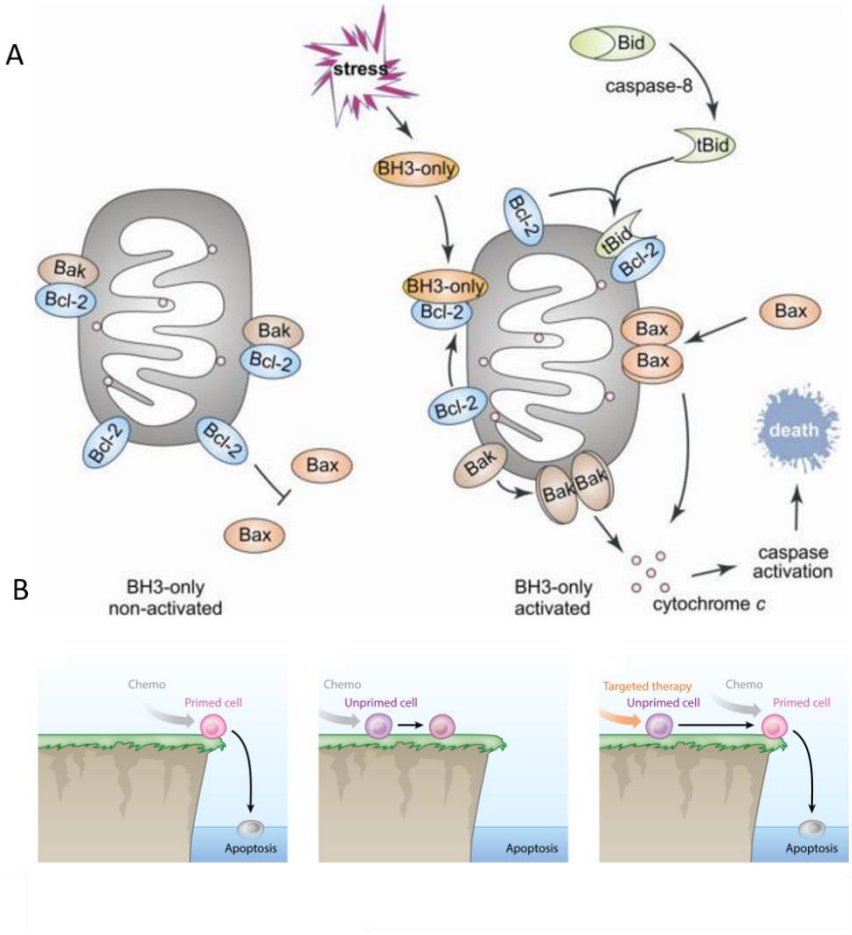

***Figure 11**: Mitochondrial pathway of apoptosis. **A)** Schematic of interactions between anti-apoptotic BCL-2 family proteins and pro-apoptotic proteins. Left: basal condition: the pore-forming units Bak and Bax are held in check by BCL-2 proteins. Right: Stress situation, the cell upregulates BH-3 only proteins to sequester anti-apoptotic proteins and to activate Bax/Bak dimerization leading to release of cytochrome c into the cytosol. Adapted from[252] **B)** Model of cells being primed for apoptosis through high-levels of oncogenic stress. Left: primed cells can easily be "pushed" into apoptosis by chemotherapy. Center: Cells high in anti-apoptotic proteins are unprimed and chemotherapy does not induce cell death. Right: Through targeted therapy with BH3-mimetics the formerly unprimed cell becomes primed and thus sensitive to chemotherapy. Adapted from[209]*

To find the individual dependency of each cancer on certain anti-apoptotic BCL2 proteins and thus their susceptibility to the respective BH3-mimetics, Letai and colleagues designed a screening approach called "BH3 profiling".[253,254] Here, they isolate mitochondria from patient cancer cells and treat them with

BH3 domain peptides. Induction of MOMP allows identification of the cancer cell's specific sensitivity towards certain BH3-mimetics.[255–257]

In other studies, senescence has been proposed as a mechanism to evade cell death and has been implicated in the adverse effects of certain chemotherapeutics and the recurrence of cancers. However, senescent cells have been shown to be dependent on specific BCL2 proteins, especially BCL-X_L. Here, BH3-mimetics have been proposed to act as senolytic agents to induce cell death in the senescent cells.[258–260]

4.4.6.2 Immunogenic cell death

Apart from apoptosis, which is considered silent in terms of evoking immune responses, other cell death modalities such as necroptosis or pyroptosis result in the release of intracellular contents. Certain molecules among these contents, so called danger-associated molecular patterns (DAMPs), can alert the immune system and stimulate its response against cell-antigens. Immunogenic DAMPs have been found to be ATP and the chromatin associated protein high-mobility group box 1 (HMGB1), but also exposure of calreticulin (CRT) on the plasma membrane due to ER stress.[261–264] Due to its immunogenic capability, these cell death modalities are also called immunogenic cell death (ICD). In cancer therapy, the idea is that after treatment-induced ICD, tumor-infiltrating dendritic cells take up the debris from cancer cells, process them and present them, in this way stimulating a systemic, adaptive immune response against cancer cells, similar to a vaccination.[265] In doing so, ICD is thought to contribute to enhanced anti-cancer effects of certain chemotherapeutic drugs. Indeed, studies could show that an increased ratio of $CD8^+$ cytotoxic T-cells (CTLs) to regulatory T-cells (T_{regs}) after chemotherapy predicts increased therapeutic responses in patients with colorectal and breast cancer.[266–270] Furthermore, lymphopenia has been shown to negatively affect the outcome in different solid tumors.[266] Of the conventional chemotherapeutics, anthracyclines have been shown to be very effective in inducing ICD in patients.[265] Further knowledge of which DAMPs are released, and which drugs induce ICD, can contribute to the development of new combination therapies strengthening the anti-cancer activity of the immune system. A study analyzing 1040 FDA-approved drugs for their propensity to enhance ICD found that cardiac glycosides were particularly effective in upregulating the DAMPs required for ICD.[271] Another approach in this respect is, to combine epigenetic modulators like histone deacetylase (HDAC) inhibitors with common therapies to induce upregulation of the DAMPs described above, thus bolstering the effects of ICD.[272]

Thus, knowledge of ICD provides tremendous opportunities to enhance chemotherapy efficacy by killing cancer cells and vaccinating the immune system.

4.5 AURORA KINASES

Aurora kinases are a family of serine/threonine kinases involved in cell division. Originally, the *aurora* allele was discovered in a *Drosophila melanogaster* mutant (*aur*) showing defective spindle-pole behavior.[273] The family was named after the night-sky phenomenon occurring close to the poles.[274] A fitting name, as Aurora kinases localize at the poles of the cell during mitosis (**Figure 12A**).

The mammalian Aurora kinase family comprises the three paralogues Aurora A, B, and C (gene names in humans: *AURKA, AURKB, AURKC*) of which AURKA corresponds to *aurora* missing in the *aur* Drosophila mutant.[275,276]

While the three mammalian paralogues differ in length and the amino-terminal sequence, the catalytic domains of Aurora A and B share 71% amino acid sequence identity (**Figure 12B**).[277] In spite of their homology, Aurora kinases have distinct localizations (**Figure 12A**) and functions. Interestingly, studies could show that these differences in location and function of Aurora A and B can be pinpointed to a single amino acid. Substitution of Gly198 in Aurora A by asparagine, the corresponding amino acid in Aurora B, led to Aurora A taking over functions of Aurora B. This was reflected by Aurora A binding to Aurora B binding partners and rescuing Aurora B knock-out phenotypes.[278,279]

Aurora A functions primarily during mitotic entry, centrosome maturation and separation, as well as in determining spindle bipolarity. It associates with the spindle poles.[280,281] Aurora B is part of the chromosomal passenger complex (CPC) which, until metaphase, associates with the inner centromere and is transferred to the spindle midzone in late mitosis. It is thought to regulate chromosome interaction with microtubules, spindle stability, and cytokinesis.[282] [281]. Aurora C closely resembles Aurora B but is primarily expressed in testis, indicating an important role in meiosis.[283]

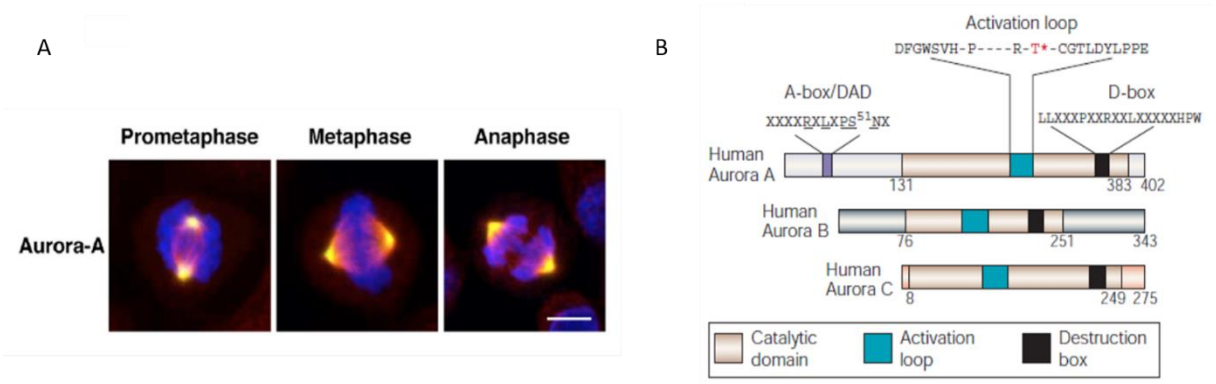

***Figure 12**: Aurora A. **A)** Subcellular localization of Aurora A at different points of mitosis. Adapted from[280] **B)** Schematic comparing Aurora A, B, and C. Adapted from[274]*

4.5.1 Interactors

4.5.1.1 Posititve regulators

One of the best studied co-factors of Aurora A is the protein TPX2. During mitosis, Ran-GTP is activated in the vicinity of chromosomes, creating a gradient of active protein close to mitotic chromosomes.[284–286] Active Ran-GTP leads to the release of microtubule-assembly factors, i.e. TPX2.[287–289] TPX2 binds Aurora A and through conformational changes allows for its activation by auto-phosphorylation of T288 in the the Aurora A activation loop. Moreover, binding of TPX2 to Aurora A protects the kinase from being inactivated by the protein phosphatase PP-1.[290,291] This TPX2 activity is important to target Aurora A to the mitotic spindle[292] where it in turn activates factors important for chromatin-driven bipolar spindle assembly.[293–295]

A second important interactor is the Borealis protein (Bora), the function of which is crucial in the role of Aurora A in mitotic entry (see below).[296]

Aurora degradation

During mitotic exit, even while they are still regulating crucial steps of the exit, Aurora kinase A and B are targeted to proteasomal degradation through the anaphase promoting complex/cyclosome (APC/C). APC/C is a multi-subunit E3-ubiquitin ligase and needs the specificity factor Cdh1.[297–300]

4.5.2 Functions in cell cycle progression

Aurora kinases play important roles in the progression of cell cycle. Their expression is upregulated transcriptionally during late S and G2 with protein levels and activity peaking at G2 and mitosis.[277,301] During G1 their expression is repressed through CDE/CHR elements.[302,303] This specific expression and the activity pattern in the G2 and M phase indicate the important role auf Aurora kinases during G2/M transition and mitosis. In the next paragraphs the diverse roles of Aurora A and its interactors are highlighted in detail.

4.5.2.1 Centrosome maturation and separation

During Interphase, centrosomes duplicate through semi-conservative duplication of the centrioles. Maturation of centrosome maturation starts in G2. Through the interplay of the kinases Pak1, Plk-1 and Cdk-11, Aurora A accumulates close to the centrosome whereas Pak1 phosphorylates Aurora A at Thr288 in the activation loop the kinase.[304–306] [307–310] This accumulation causes the growth of the pericentrosomal material (PCM), increasing centrosomal microtubule nucleation activity.[281] Aurora A in turn orchestrates and activates components for centrosome maturation, i.e. centrosmin[311] or NDEL1[312]. Furthermore Aurora A also activates proteins important for microtubule nucleation such as LATS[313,314] or TACC proteins[315].

4.5.2.2 Mitotic entry / G2 checkpoint recovery

Mitotic entry is controlled directly by the mitotic cyclin-B and the cyclin-dependent kinase 1 (Cdk1), as well as the phosphatase cdc25.[316] During S and G2 phase the catalytic activity of Cdk1 is inhibited by phosphorylation through the kinases Wee1 and Myt1. This phosphorylation is removed by cdc25. Active cylin-B/Cdk1 in turn, inhibits Wee1 and Myt1 and activates cdc25, thus creating a positive, so called “inner” feedback loop.[317,318]

The “outer” feedback loop comprises several feedback mechanisms, most importantly through Polo-like kinase-1 (Plk-1) and Aurora A, which strengthen the inner loop (**Figure 13A**). Here, Aurora A promotes the mitotic entry by controlling the Plk1-dependent activation of Cyclin-B/Cdk1.[319] Aurora A phosphorylates T210 in the Plk-1 kinase domain thus activating it.[320] A critical co-factor for this reaction is the Borealis protein (Bora). Binding of Bora to the phosphate-binding domain (PBD) of Plk-1 interferes with the intramolecular interaction of the PBD and kinase domain which renders T210 accessible for Aurora A-mediated phosphorylation (**Figure 13B**).[296] Active Plk-1 phosphorylates Cdc25c, increasing nuclear accumulation of the phosphatase. Plk-1 can further phosphorylate Myt1 and Cdk1-phosphorylated Wee1 leading to its degradation.[318,321,322] Thus, Aurora A, through Plk-1[296], facilitates the inner feedback loop, shifting the equilibrium towards active Cyclin B/Cdk1. Active Cdk1 contributes

to this feedback loop by, on the one hand stabilizing Bora and enhancing its binding to Plk-1, allowing for efficient Aurora A-mediated activation of Plk-1.[323,324] On the other hand Cdk1 also regulates the function of the phosphatase PP1, which is known to inactivate Aurora A through dephosphorylation of T288.[319,325,326]

Aurora A in turn, can also directly influence the inner feedback loop through phosphorylation of Cdc25b.[327]

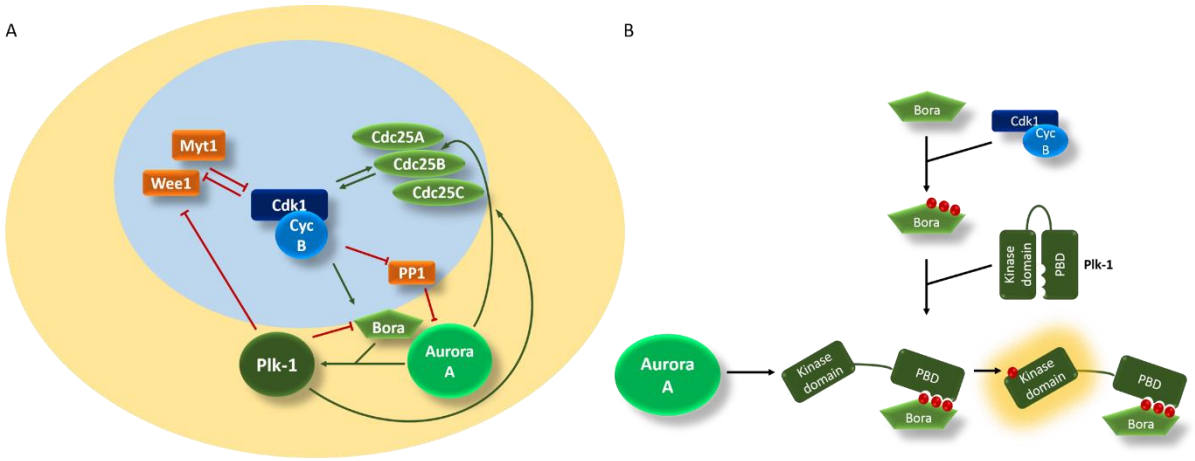

***Figure 13**: Regulatory loops involving Aurora A. A) Inner (blue) and outer (yellow) loop of CDK1 activation. Green: positive regulators, orange: negative regulators. B) Aurora A-dependent regulation of PLK-1 activity. Adapted from[328]*

While the inner feedback loop is the major driver of mitotic entry, the regulation through the outer feedback loop with Aurora A and Plk-1 shows redundancy and seems to be not essential. Depletion of single factors has no or little effect on mitotic entry but leads to abnormal cell division and mitotic abnormalities. These findings underscore the redundant role of many factors for mitotic entry but also their critical role in the orchestration of mitotic events. However, while factors like Aurora A, Plk-1 or Cdc25 are redundant in normal mitotic entry, their specific activity is essential for G2 checkpoint recovery.[296,318,325,329,330]

The G2 checkpoint is the last instance for a cell to avoid transmission of mutations to the daughter cells:[331] DNA damage or stalled replication forks after S-phase are sensed by the proteins ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related protein (ATR), which activate checkpoint kinases (Chk) 2 and 1, respectively. Chk1/2 regulate p53 activity and DNA-repair pathways.[332] p53 transcriptionally represses cyclin B expression.[331] Moreover, Chk-dependent phosphorylation stabilizes Wee1, destabilizes Cdc25A, and sequesters Cdc25C in the cytoplasm, resulting in reduced Cyclin B/Cdk1 activity (**Figure 14A**). Due to these activities, the inner feedback loop is halted, and mitotic entry is prevented. This so called G2/M arrest allows for efficient DNA repair.[331] If the DNA damage burden is too high and exceeds the capacity of repair mechanisms, cells are removed from cell cycle in a p53-dependent manner, either by entering senescence or by undergoing intrinsic apoptosis.[331,333] In many cases however, DNA lesions are efficiently repaired and cells can safely re-enter the cell cycle in a process called checkpoint recovery.

In this process, Plk-1 activity mediated by Aurora kinase A plays a crucial role. During DNA damage, Plk-1 activity is low. For checkpoint recovery Aurora kinase A activity increases and thereby regulates

Plk-1 activity. Plk-1 in turn targets both Wee1 and Claspin (an important co-factor of ATR) for degradation and has been found to directly inhibit Chk2. Together with the effects of Plk-1 and Aurora kinase A on Cdc25 activity (**Figure 14B**), these mechanisms result in re-activation of Cyclin B/Cdk1 activity, thus allowing for the G2/M-transition. Due to the drastic alterations during cell cycle arrest compared to normal progression, protein balance and activity is still perturbed after checkpoint recovery.[333,334] In this scenario activity of Aurora kinase A and Plk-1 is essential.[331]

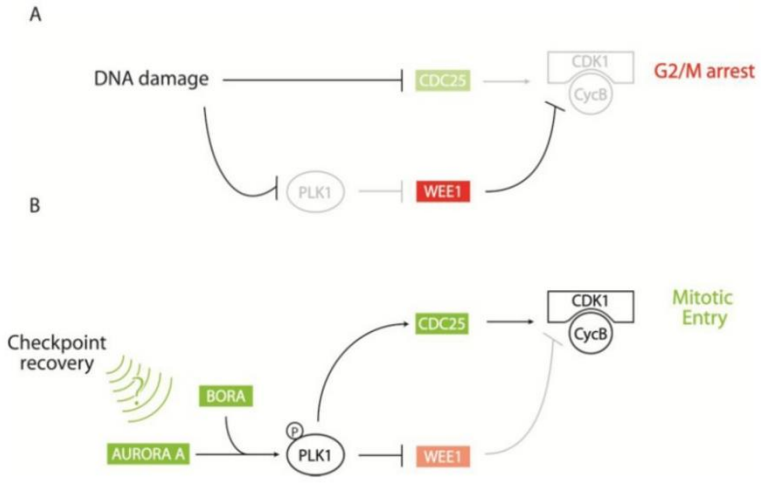

Figure 14: *Roles of Aurora A and PLK1 in recovery from G2/M arrest. A. G2/M checkpoint: The DNA damage response inhibits PLK1 and Cdc25 and Wee1 inhibits Cyclin B/CDK1 to induce a G2/M arrest. B. Checkpoint recovery: After successful DNA repair, PLK1 is activated in an Aurora A-dependent manner, allowing for mitotic entry. Adapted from*[335]

4.5.2.3 Bipolar spindle assembly / M checkpoint control

After maturation, in late G2 the centrosomes migrate to define the two poles of the bipolar mitotic spindle. The phenotype of the *Drosophila aurora* mutants and other models systems showed high frequency of monopolar spindles indicating a crucial role of Aurora kinase A not only for centrosome maturation but also for segregation.[273,336–338] Studies in *C. elegans* showed that upon RNAi-mediated silencing of Aurora kinase A, centrosomes indeed first separated but later collapse back.[339] [336] Further studies indicate that Aurora A affects the occurrence of astral microtubules which connect centrosomes to the cell cortex and are important for spindle bipolarity.[340]

The M checkpoint, also called spindle assembly checkpoint (SAC), is important during mitosis, in the prometaphase.[341] It is activated when kinetochores of the chromosomes are not properly attached to the mitotic spindle: Unattached kinetochores facilitate the formation of the mitotic checkpoint complex (MCC) that blocks activity of the APC/C. This lack of APC/C activity results in the cohesin rings around sister chromatids to remain closed, thereby preventing segregation, thus halting the mitotic process.[341] The process of “switching on” the SAC relies on Aurora kinase B as part of the CPC.[341,342] While the role for Aurora kinase B in SAC-control has been established, the role of Aurora kinase A for SAC activity remains controversial.[343] Some studies demonstrated that Aurora kinase A is dispensable for SAC

activity.[344,345] Other studies, in turn, could show that Aurora kinase A regulates SAC inactivation[346] and Aurora B feedback loops.[343]

4.5.3 Aurora Kinases in cancer

4.5.3.1 Oncogenic potential of Aurora A

The *AURKA* gene is located on a chromosomal region known to be intrinsically unstable, frequently mutated and amplified in many tumors.[347] Among the first reports on Aurora A functions were studies describing its overexpression and oncogenicity in colorectal cancer[277] and breast cancer.[348,349] The latter study also coined one of the other names of Aurora A: breast tumor activated kinase (BTAK).[349]
In the past decades Aurora A has been found to be overexpressed or mutated in a variety of different cancers such as lung, cervical, prostate, and oral cancer, melanoma, glioma, and acute myeloid leukemia (AML).[347,350]

Regarding function, increased activity of Aurora kinase A can drive premature G2 checkpoint recovery resulting in mutations being more frequently passed on, thereby promoting tumorigenesis. Consequently, overexpression of Aurora A has been shown to promote colony formation, centrosome amplification, chromosomal instability, and tumor growth in mouse xenograft studies.[277,349,351,352] While amplification of Aurora A has been shown to be correlated with the p53 status of the cancer cell, tetraploidisation as a result of centrosome amplification also is p53-dependent.[351,353] Furthermore, Aurora kinases are implicated in resistance of tumors against chemotherapy (i.e., taxols or cis-platin)[354,355] or radiotherapy[356].

While overexpression of Aurora A alone is sufficient to transform certain cells, the process is not consistent, indicating that additional oncogenic events are required, for example aberrant Ras-signaling.[357] The exact contribution of Aurora A to transformation has not been solved yet, although there is evidence that Aurora A interferes with spindle assembly checkpoint (SAC) activity, thus increasing genome instability.[355] Furthermore, Aurora A overexpression promotes EMT in transformed cells.[358–360] In this way, Aurora A overexpression contributes to malignancy and results in worse prognosis for patients with hepatocellular carcinoma or gastric cancer.[361,362]

4.5.3.2 Interaction of Aurora A with tumor suppressors

Aurora A can directly phosphorylate p53 at Ser215 and Ser315, and thus control p53 stability and transcriptional activity.[346,350,363] p53 in turn, can inhibit Aurora A function by directly binding to the catalytic domain.[364] Moreover, Aurora A has been shown to phosphorylate BRCA1 at Ser308 while located at the centrosome suggesting that disruption of the G2/M transition by BRCA1 and Aurora A predisposes to carcinogenesis.[365]

4.5.3.3 Interaction of Aurora A with oncogenes

Aurora A expression can be upregulated by c-myc, which in turn can upregulate myc expression, creating a positive feedback loop.[366,367] Also, as shown in **Figure 15**, Aurora A stabilizes n-myc by protecting it from FBXW7-mediated degradation.[368–370]

In ovarian and breast cancer, Aurora A has been linked to the RAS/RAF/MEK/ERK pathway, through which it stimulates telomerase activity in a c-myc-dependent manner.[371] Later it could be shown that the small GTPase RalA is a direct target of Aurora A phosphorylation, linking the kinase to the Ras pathway.[372] Aurora A can also modulate Ras signaling through phosphorylation of the tumor suppressor RAS-association domain family 1, isoform A (RASSF1A), thereby disrupting microtubule stabilization and M-phase cell cycle arrest.[373]

Furthermore, it could be shown that Aurora A inactivates apoptotic signaling through upregulation of NFkB activity.[374] Aurora A was also shown to modulate the equilibrium of anti-apoptotic (Bcl-2, Mcl-1) and pro-apoptotic proteins (Bax, Bim, PUMA) at the mitochondrial membrane.[375–378]

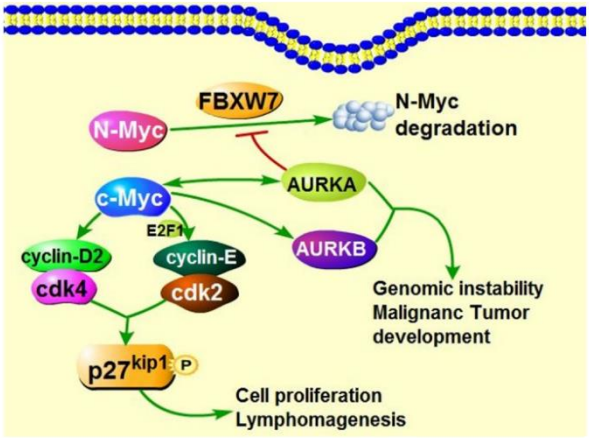

***Figure 15**: Interaction of Aurora kinases with MYC oncogenes and the influence on tumorigenesis. Adapted from[347]*

4.5.3.4 Aurora A as a therapeutic target in cancer

Pre-clinical data shows that inhibition of Aurora A leads to suppression of cell proliferation, migration and invasion.[347,379–381] Treatment of tumor cells with a selective Aurora A inhibitor resulted in defective bipolar spindle assembly and chromosome separation. As a result, cancer cells either underwent mitotic arrest and when exiting mitosis showed aneuploidy and centrosome amplification[382], or Aurora A inhibition induced senescence[383]. Mitotic arrests after combination with spindle poisons indicate a compromised SAC.[384]

In the past decade, first clinical trials have started with different inhibitors (see **Table 1**). The most promising of them is MLN8237 (Alisertib) which has shown mild effects on solid tumors.[385]. In hematological cancer it was already studied in a phase III clinical trial.[386].

Clinical trials so far show that single-agent treatment against Aurora A has modest effects and is still subject to toxicity.[347,387] However, due to the contribution of Aurora kinases to therapy resistance, Aurora kinase inhibition has been proposed to work in combination with conventional therapies. Indeed, pre-clinical data shows synergy of Aurora A inhibition in combination with Paclitaxel in pancreatic cancer cells[388] or in combination with cis-platin[389]. Aurora kinase inhibition can sensitize androgen-resistant prostate cancer or atypical teratoid rhabdoid tumor cells to radiotherapy.[390–393] Moreover, targeted therapy approaches such as the use of monoclonal antibodies proved to be effective in combination with Aurora A inhibition. It could be shown that Aurora A inhibition overcomes cetuximab-resistance in squamous cell cancer of the head and neck.[394]
In a recent high-throughput study using genetic and pharmacological compound screens, Wang and colleagues could show that Aurora kinase inhibition leads to senescence in Ras-mutated lung cancer and melanoma cell lines, thus describing another mechanism of action of Aurora inhibition. [395] Furthermore, that study could show that cells rendered senescent through Aurora kinase inhibition, become susceptible to treatment with BH3-mimetics which act as senolytic agents. Wang and colleagues thus proposed a combination therapy of Aurora kinase inhibitor as a senescence inducer and ABT-263 as a senolytic drug.[395]

While the role of Aurora kinases has been extensively investigated in various carcinomas or hematological diseases, only few studies looked at Aurora kinases in sarcomas. Pre-clinical studies showed anti-tumor effects of Aurora kinase A inhibition in xenograft models of liposarcoma[396] or human synovial sarcoma[397]. Furthermore, Aurora kinase A has been investigated as a target in osteosarcoma and proposed as a tumor marker.[398,399]
Research of Aurora kinases in pediatric malignancies mainly focused on hematological diseases or neuroblastoma. The pediatric pre-clinical testing program (PPTP) tested Aurora kinase A inhibition in 2010 in a variety of their xenograft models. They could show complete responses in almost half of their neuroblastoma models, all of their acute lymphoblastic leukemia models, and a variety of other tumor models, featuring also rhabdomyosarcoma, Ewing's sarcoma, and osteosarcoma models.[42].
In 2011 a pre-clinical study has been conducted, showing that Aurora A inhibition reduced proliferation of MYCN-amplified childhood neuroblastoma cells and decreased tumor growth in a MYCN neuroblastoma mouse model.[400]
Despite promising pre-clinical data, in recent years only four clinical trials have been conducted investigating Aurora kinase inhibition in childhood malignancies. Currently there is one study recruiting patients for a phase II study of Alisertib in rhabdoid tumors (*https://clinicaltrials.gov* NCT02114229).

***Table 1**: Current status of Aurora kinase inhibitors in clinical trials. Data obtained from* https://clinicaltrials.gov in June 2016. *Table adapted from*[401]

Name	Clinical trial	Disease condition	Starting date	Sponsors	Current status
Alisertib (MLN8237)	Phase 3 NCT01482962	Relapsed or refractory peripheral T-Cell lymphoma	June 2012	Millennium pharmaceuticals, inc. Takeda	Ongoing
	Phase 2 NCT02560025	Induction Chemotherapy in patients with High-risk acute myeloid Leukaemia	December 2015	Millennium pharmaceuticals, inc. Takeda	Ongoing
	Phase 2 NCT01316692	Patients with unresectable stage iii-iv melanoma	October 2011	Vanderbilt-ingram cancer centre	Terminated (April 2016)
	Phase 2 NCT02038647	Small-cell-lung Cancer (SCLC) treatment in combination with paclitaxel	February 2014	Millennium pharmaceuticals, inc. Takeda	Ongoing
	Phase 1 NCT02551055	Neoplasms, Advanced or Metastatic	October 2015	Millennium pharmaceuticals, inc.|takeda	Ongoing
	Phase 1 NCT02214147	Advanced solid tumors or Relapsed/Refractory lymphoma	September 2014	Millennium pharmaceuticals, inc	Completed
Tozasertib (VX-680, MK0547)	Phase 2 NCT00290550	Non-small-cell lung carcinoma	June 2006	Merck sharp & dohme corp.	Terminated
	Phase 2 NCT00405054	Leukaemia	December 2006	Merck sharp & dohme corp.	Terminated
	Phase 1 NCT00500006	Chronic myelogenous leukaemia, lymphoblastic, acute, philadelphia-positive	October 2007	Merck sharp & dohme corp.	Terminated
	Phase 1 NCT00111683	Chronic Myelogenous leukaemia in blast crisis, lymphocytic leukaemia, B Cell acute and chronic myelogenous leukaemia	June 2005	Merck sharp & dohme corp.	Completed August 2015
	Phase 1 NCT02532868	Advanced solid tumors	May 2005	Merck sharp & dohme corp.	Terminated
	Phase 1 NCT00099346	Colorectal cancer	January 2005	Merck sharp & dohme corp.	Terminated
Berasertib (AZD1152)	Phase 3 NCT00952588	Acute myeloid leukaemia	July 2009	Astrazeneca	Completed February 2014
	Phase 2 NCT01354392	Diffuse large B-cell lymphoma	September 2011	Oxford university hospitals nhs trust	Completed July 2014
	Phase 1 NCT00338182	Advanced solid malignancies	May 2006	Astrazeneca	Completed March 2017
	Phase 1 NCT00497991	Patients with relapsed acute myeloid leukaemia	May 2006	Astrazeneca	Completed December 2010
	Phase 1 NCT00497731	Advanced solid malignancies-study 3	August 2006	Astrazeneca	Terminated
MLN8054	Phase 1 NCT00249301	Advanced solid tumors	October 2005	Millennium pharmaceuticals, inc takeda	Terminated
	Phase 1 NCT00652158	Advanced malignancies	April 2006	Millennium pharmaceuticals, inc Takeda	Terminated
Danusertib (PHA739358)	Phase 2 NCT00872300	Multiple myeloma	October 2008	Nerviano medical sciences	Completed May 2014
	Phase 2 NCT00766324	Hormone refractory prostate cancer	September 2007	Nerviano medical sciences	Completed May 2014
AT9283	Phase 2 NCT01145989	Patients with relapsed or refractory multiple myeloma	June 2010	Ncic clinical trials group	Completed November 2015
	Phase 2 NCT00522990	Leukaemia	September 2006	Astex pharmaceuticals	Terminated
	Phase 1 NCT00985868	Relapsed and Refractory Solid Tumors	September 2009	Cancer research uk	Completed April 2013
	Phase 1 NCT01431664	Patients With Relapsed or Refractory Acute Leukaemia	September 2011	Cancer research uk	Completed December 2014
	Phase 1 NCT00443976	Advanced or metastatic solid tumors or non-hodgkin's lymphoma	January 2007	Ncic clinical trials group	Completed January 2012

Name	Clinical trial	Disease condition	Starting date	Sponsors	Current status
AMG-900	Phase 1 NCT00858377	Advanced solid tumors	August 2009	Amgen	Ongoing
	Phase 1 NCT01380756	Acute myeloid leukaemia	July 2011	Amgen	Completed February 2015
VX-689 (MK-5108)	Phase 1 NCT00543387	Advanced and/or Refractory solid tumors	March 2008	Merck sharp & dohme corp.	Completed April 2011
TAK-901	Phase 1 NCT00807677	Advanced hematologic malignancies	March 2009	Millennium pharmaceuticals, inc. Takeda	Completed March 2013
	Phase 1 NCT00935844	Advanced solid tumors or lymphoma	October 2009	Millennium pharmaceuticals, inc. Takeda	Completed November 2011
GSK1070916	Phase 1 NCT01118611	Advanced solid tumors	March 2010	Cancer research uk	Completed March 2013
ENMD-2076	Phase 2 NCT01914510	Ovarian clear cell cancers	September 2013	University health network, toronto	Ongoing
	Phase 2 NCT01639248	Previously treated locally advanced + metastatic tnbc	July 2012	Casi pharmaceuticals, inc	Ongoing
	Phase 2 NCT02234986	Advanced fibrolamellar carcinoma	October 2015	Casi pharmaceuticals, inc.	Ongoing
	Phase 2 NCT01104675	Patients with ovarian cancer	April 2010	Casi pharmaceuticals, inc.	Completed December 2012
	Phase 1 NCT00658671	Advanced cancer	April 2008	Casi pharmaceuticals, inc.	Completed June 2012
	Phase 1 NCT00806065	Multiple myeloma	December 2008	Casi pharmaceuticals, inc.	Completed January 2012

5. AIM OF THE THESIS

As described above, the overall survival of patients with aRMS is still low, especially in disseminated disease. The standard of care therapy is harsh and comes with many side effects for the young patients. To reduce the burden of therapy side effects and increase overall survival in these patients, we need to find new strategies to target aRMS.

To this end, we focused on interfering with the biology of the pathognomonic fusion protein. The therapeutic potentital of targeting PAX3-FOXO1 activity has been demonstrated before in different approaches of our group.[137,150] Earlier studies could already demonstrate that genetic depletion of PAX3-FOXO1 leads to cell death in aRMS, presumably by apoptosis.[99] However, the exact mechanism of cell death has not been characterized so far.

Hence, in this thesis we used unbiased drug screens and genetic means to unravel the exact mode of cell death, the role of PAX3-FOXO1 in preventing it, and to convert these finding into a new targeted combination therapy approach.

The aims of this thesis are as follows:

1) Characterize cell death in aRMS cells after loss of PAX3-FOXO1 and find a combination therapy interfering with fusion protein biology while simultaneously enhancing cell death. (Manuscript I)
2) Investigate the PAX3-FOXO1-dependent regulation of NOXA in aRMS and its implications for new therapy approaches. (Manuscript II)

6. RESULTS

6.1 MANUSCRIPT I

Aurora A kinase inhibition destabilizes MYCN and PAX3-FOXO1 and synergizes with Navitoclax to control Rhabdomyosarcoma tumor growth

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Manuscript ready for submission

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Author contributions: JO, JLS, MW, DL, MR, PKB, and AK acquired data. JO, JLS, MW, PKB, and AK contributed to analysis and interpretation of the data. JO, MW, JS, and BWS made substantial contributions to the conception, design, and intellectual content of the paper. JO and BWS wrote the paper.

Abstract

Rhabdomyosarcoma is the most common soft tissue sarcoma in children. The aggressive alveolar subtype (aRMS) is characterized by chromosomal translocations, most often a t(2;13) resulting in the expression of the oncogenic fusion protein PAX3-FOXO1 which is critical for tumorigenesis and cell survival.

Our aim here was to identify a pharmacological combination therapy approach interfering with PAX3-FOXO1 biology at different levels. Since loss of the fusion protein results in cell death, we first aimed to pharmacologically enhance this effect. To this end, we screened aRMS tumor cells with a library of 208 drugs while simultaneously silencing PAX3-FOXO1 by shRNA. This identified the BH3-mimetic ABT-263 to sensitize aRMS cells to cell death after PAX3-FOXO1 depletion. To further characterize the cell death mechanisms we used combined shRNA and CRISPR approaches to perform a BH3 protein profiling. In accordance with identification of ABT-263 we demonstrate that aRMS cells undergo intrinsic apoptosis in a NOXA-dependent manner upon depletion of PAX3-FOXO1. In a parallel approach, and to identify drugs altering PAX3-FOXO1 protein stability, we screened the same drug library directly measuring fusion protein levels as read-out. This revealed that inhibition of Aurora kinase A negatively affects PAX3-FOXO1 protein levels. Furthermore, we demonstrate that Aurora kinase A stabilizes MYCN, both an oncogene and target gene of PAX3-FOXO1 in aRMS, and that inhibition of the kinase also destabilizes MYCN.

Finally, using both aRMS cell lines and patient-derived xenografts we demonstrate that combination treatment of Aurora kinase A inhibitors together with ABT-263 synergistically induces cell death and greatly slows tumor growth *in vitro* and *in vivo*.

Taken together, these data show a novel functional interaction of Aurora kinase A with both PAX3-FOXO1 and its effector MYCN, and suggest new opportunities for targeted combination treatment of aRMS.

Keywords: PAX3-FOXO1, MYCN, drug screening, BH3-mimetics, Aurora kinase A, apoptosis

Introduction

Rhabdomyosarcoma is the most common pediatric soft tissue sarcoma. The most aggressive subtype, alveolar rhabdomyosarcoma (aRMS), is characterized by the occurrence of balanced reciprocal translocations resulting in the expression of oncogenic fusion proteins [1,2]. The most common fusion, PAX3-FOXO1, is the result of a translocation between chromosomes 2 and 13. aRMS has a high propensity to metastasize and resistances to standard-of-care treatments are common, resulting in the worst prognosis for all rhabdomyosarcoma types, with 5-year survival rates of only about 30% [3]. The search for novel targeting strategies is difficult as pediatric tumors generally harbor fewer somatic mutations than adult tumors [4]. Especially the aRMS subtype harbors very few mutations as opposed to other rhabdomyosarcoma subtypes [5]. The lack of other somatic mutations underscores the important role of PAX3-FOXO1 in aRMS tumor development and maintenance. Its function as transcriptional activator results in activation of multiple oncogenic pathways [6]. Our group showed that antisense-mediated loss of PAX3-FOXO1 results in cell death, underlining the addiction of aRMS cells to the fusion protein. However, so far the exact mechanism by which the cells die has not been described [7].

Due to its importance in tumor survival, targeting PAX3-FOXO1 has become a paramount goal in rhabdomyosarcoma research in recent years. However, since PAX3-FOXO1 is a transcription factor lacking enzymatic activity, targeting the fusion protein directly is challenging. Recently, new strategies have been developed targeting PAX3-FOXO1 indirectly by inhibiting its stabilizer Polo-like kinase 1 (PLK1) [8] or its co-factors, like bromodomain-containing protein 4 (BRD4) [9] or chromodomain-helicase DNA-binding protein 4 (CHD4) [10]. Further strategies to target PAX3-FOXO1 are reviewed in [11]. While single-target therapies have been shown to be efficient in pre-clinical models and clinical treatment, they tend to be prone to therapy resistance. One way to circumvent this problem would be to use drugs in combination, thus targeting different pathways and making it harder for cancer cells to escape.

Aurora kinases are a family of serine-threonine kinases that are involved in cell cycle progression, most importantly during mitosis. The family consists of three homologues Aurora A, B and C encoded by the genes *AURKA*, *AURKB* and *AURKC,* respectively [12]. Each kinase serves distinct and tightly regulated functions during mitosis or meiosis. Aurora kinase A is highest expressed at the G_2/M transition [13]. Here, its function is to phosphorylate PLK1 at threonine 210, thus activating it which is a crucial step for checkpoint recovery [14,15]. Furthermore, Aurora kinase A serves important roles in centrosome

maturation and mitotic entry [16]. During prometaphase it also localizes to spindle poles facilitating bipolar spindle assembly [17]. These functions for cell cycle progression indicate an important role for Aurora kinases in cancer. Indeed, Aurora kinase A has been found to be upregulated in a variety of tumors and has thus been a focus of novel preclinical therapy approaches in the recent years [18–21]. Apart from its mitotic function, Aurora kinase A has been shown to phosphorylate AKT and mTOR indicating a role in promoting resistance of cancer cells towards chemotherapy [22].

Its close relation to PLK1 also makes Aurora kinase A an interesting target in rhabdomyosarcoma. Recently, we showed that PLK1 stabilizes PAX3-FOXO1 in aRMS tumors through phosphorylation of serines 503 and 505, protecting the fusion protein from proteasomal degradation [8]. While single-agent inhibition of PLK1 proved very effective in reducing tumor growth in vivo, resistance against this treatment was common. Our goal was thus to find a novel effective combination therapy approach to treat aRMS.

To this end, we first aimed to characterize the exact mode of cell death after silencing of PAX3-FOXO1. Then, based on our findings, we aimed to find drugs enhancing this mode of cell death as well as more drugs interfering with PAX3-FOXO1 biology. Our goal was, we wanted to find a novel synergistic combination.

Here, we demonstrate that aRMS cells undergo intrinsic apoptosis in a NOXA-dependent manner after loss of PAX3-FOXO1. Using a drug library, we found that BH3-mimetics, especially ABT-263, can efficiently enhance cell death in a NOXA-dependent fashion. Moreover, we established a novel functional link between Aurora kinase A and PAX3-FOXO1 stability. Combination of ABT-263 and the Aurora A inhibitor Alisertib have synergistic effects tumor in vitro and in vivo and thus provide the basis for a promising new combination therapy approach in aRMS treatment.

Results

Silencing of PAX3-FOXO1 expression induces intrinsic apoptosis in a NOXA-dependent manner

Since it was previously shown that silencing of PAX3-FOXO1 expression results in FP-RMS cell death (Bernasconi et al., 1996), we aimed first to further elucidate the precise mechanism of this cell death.

To this end, we generated FP-RMS cell lines expressing an inducible shRNA (shP3F) or control (shsc) to specifically silence PAX3-FOXO1 expression upon doxycycline treatment (Suppl.Fig.1A). After confirming that the system significantly downregulates the fusion protein both on the mRNA and protein level in four different cell lines (Suppl.Fig.1B-D), we assessed whether cells would undergo apoptosis. We observed a significant increase in caspase 3/7 activity in Rh4 and Rh30 cells 48 hours and 72 hours after P3F depletion compared to control (Fig.1A, Suppl.Fig.1E). This increase was also reflected on the protein levels of the cleaved products of PARP, caspases 3, 7, and 9 after 48h of shRNA induction (Fig.1B). To exclude off-target effects of the shRNA, we overexpressed a non-targetable PAX3-FOXO1 mutant which indeed could rescue cells from apoptosis despite silencing of the endogenous fusion protein as shown by reduced caspase 3/7 activity (Suppl.Fig1F) and decreased levels of cleaved PARP and caspase 3 protein products (Suppl.Fig1G).

To further confirm this notion, we also treated cells after fusion protein depletion with increasing concentrations of the pan-caspase inhibitor zvad-FMK which indeed could restore viability of Rh4sh cells (Fig.1C) and reduce cleaved PARP and caspase 3 protein levels (Suppl.Fig1H). To exclude other modes of cell death, we treated shP3F-Rh4 cells with different cell death inhibitors. Interestingly, 48h fusion protein depletion only zvad-FMK could rescue viability but none of the other inhibitors (Fig.1D). These data indicate that apoptosis is the major mode of cell death activated in FP-RMS cells upon depletion of PAX3-FOXO1.

Next, we sought to identify the pro-apoptotic protein(s) responsible for initiating apoptotic cell death. To this end, we performed a small scale CRISPR/Cas9 screen in shP3F-Rh4 cells using the construct depicted in Suppl.Fig.2A to knock-out pro-apoptotic genes either individually or in combination and measured cell viability upon P3F depletion. Knock-down efficiencies of Bax, Bak, Bad, Bim as examples are shown in Suppl.Fig.2B-C. In control cells (shsc-Rh4), only depletion of caspase 9, but not caspase 8 and the combination of Bax/Bak, were able to significantly reduce caspase 3/7 activity (Suppl.Fig.2D). This indicates that activation of the extrinsic pathway is less important in PF-RMS cells. Upon depletion of PAX3-FOXO1, NOXA was the only BH3-only protein capable to reduce caspase 3/7 activity

significantly (Fig.1E, Suppl.Fig.2E) and similar to knock out of the pore forming proteins BAX and BAK. These results were also confirmed in three additional FP-RMS cell lines (Suppl.Fig.2F) indicating that NOXA in general plays an important role in initiating apoptosis after PAX3-FOXO1 depletion. Moreover, this is in line with the observation that both mRNA and NOXA protein expression are upregulated upon silencing of the fusion protein in all cell lines tested (Fig.1F-G, Suppl.Fig.2G). Taken together, our results demonstrate that FP-RMS cells undergo intrinsic apoptosis upon silencing of PAX3-FOXO1 which depends on upregulation of the BH3-only protein NOXA.

The BH3-mimetic ABT-263 enhances cell death after PAX3-FOXO1 depletion

Next, we aimed at finding drugs that would enhance apoptosis induced by PAX3-FOXO1 depletion. For this, we set up a compound screen of 205 drugs at a final concentration of 500 nM and cotreated shP3F-Rh4 cells together with doxycycline mediating induction of fusion protein directed shRNA (Suppl.Fig.3A, Supplemental table 1). Results are depicted as ratio of viability comparing shP3F versus shsc cells. The screen identified 13 candidate drugs that decreased viability of shP3F cells by at least an additional 50% while remaining unchanged in control shsc cells (Fig.2A). Classification of the top hits according to their mechanism of action revealed that 8 out of the 13 drugs were either Aurora kinase A inhibitors (5) or BH3-mimetics (3) (Fig.2B) with ABT-263 being the most potent drug, also when validating each as single agent (Fig.2C, Suppl.Fig.3 B-M). To directly compare BH3-mimetics that act more specifically on Bcl-2 itself or more broadly on the family, we generated dose-response curves of ABT-263 and ABT-199 on shP3F-Rh4 cells (Fig.2D-E). Interestingly, while both ABT-263 and ABT-199 showed a further reduction of IC_{50} upon silencing of PAX3-FOXO1, ABT-263 reduced IC_{50} to at least ten times lower concentration (reduction of 3.0μM to 0.18μM) than ABT-199 (reduction of 9.6uM to 5.6uM). These findings indicate that inhibition of BCL-2 itself might be less important than other family members. Hence, we treated cells with increasing concentrations of the BCL-X_L specific inhibitor A1331852 and S63845 which is MCL-1 specific. While A1331852 showed comparable effects to ABT-263 (Fig.2F), while treatment with S63845 did not further increase cell death (Suppl.Fig.3O). These results support the notion that PAX3-FOXO1 silencing primes FP-RMS cells to inhibition of BCL-xl via upregulation of NOXA.

To demonstrate this directly, we treated shP3F-Rh4-NOXA$^{-/-}$ cells (Suppl.Fig.2E) with ABT-263 and found indeed reduced sensitivity upon silencing of PAX3-FOXO1 (Fig.2G). Finally, expression analysis of different datasets in the r^2 database (R2: Genomics Analysis and Visualization Platform:

http://r2.amc.nl) revealed that NOXA expression was elevated in all rhabdomyosarcoma datasets compared to healthy skeletal muscle tissue (Suppl.Fig.3P).

These findings suggest that rhabdomyosarcoma cells might be already primed towards a pro-apoptotic state through higher basal NOXA expression which can be further enhanced by reduction of fusion protein levels and subsequently renders them more sensitive towards ABT-263 treatment.

Aurora kinase A inhibition reduces PAX3-FOXO1 protein stability

In a next step, we therefore aimed to pharmacologically reduce PAX3-FOXO1 protein levels in FP-RMS cells. To this end, we treated wild type Rh4 cells with the same drug library (Supplemental Table 1) and analyzed cell lysates by Western Blot to identify drugs that have the potential to reduce PAX3-FOXO1 protein levels (Suppl.Fig.4A). To exclude that reduced fusion protein levels were due to general toxic effects all signals were densitometrically digitalized and normalized to the house keeping protein GAPDH. Hits were called when lowering fusion protein levels below 80%, a criterion fulfilled by 43 compounds (Fig.3A-B). When classifying these hits according to their drug targets we identified five epigenetic regulators, five proteasome, five Aurora kinase A, and three CDK9 inhibitors (Fig.3C). Of these, aurora kinase A caught our attention because of its ability to stabilize the NMYC protein [31]. Hence, we individually validated AURKA inhibitors at increasing doses to reduce PAX3-FOXO1 protein levels and identified alisertib as being the compound active at the lowest doses tested (Fig.3D, Suppl.Fig.4B-C). Strikingly, treatment of patient-derived primary cells with alisertib also reduced fusion protein levels (Suppl.Fig.4C).

Previous work from our laboratory demonstrated a direct interaction between PAX3-FOXO1 and the Polo-like kinase 1 (PLK1) which stabilizes the fusion protein via phosphorylation at S503 [8]. PLK1 in turn is known to be activated by AURKA [15]. Hence, we were interested to study the mechanism underlying the functional interaction of AURKA and PAX3-FOXO1. We treated Rh4 cells with alisertib and analyzed PLK1 protein levels and activity by Western Blot after 48hrs. This revealed increased protein levels of both PLK1 and Aurora kinase A. In addition, we observed a reduction of PLK1 phosphorylation at threonine 210 as expected (Fig.3E). Since AURKA has been described to influence phosphorylation of S256 in wild type FOXO1 [23], we also investigated phosphorylation of PAX3-FOXO1 at serine 437 (corresponding serine 256 in wild-type FOXO1). Indeed, we also observed a clear reduction at this site in the fusion protein upon Alisertib treatment (Fig.3E). As this region of PAX3-FOXO1 has been described to be relevant for protein stability (reviewed in [11]), we next studied whether

phosphorylation of serine 437 would contribute to fusion protein stability by replacing serine with alanine at this position (S437A). Interestingly, and even more pronounced than the S503A mutant, also the S437A mutant significantly reduced fusion protein stability compared to wild-type as revealed by 8hr treatment with cycloheximide (Fig.3F-G).

Lastly, to investigate a potential direct interaction of AURKA and PAX3-FOXO1 we fused PAX3-FOXO1 to the bacterial biotin-ligase BirA [24] and expressed it ectopically in HEK293T cells. After pull down of biotinylated proteins using streptavidin coated beads, PLK1 was identified on Western Blots to be selectively biotinylated in fusion protein samples but not in the BirA-only control as shown before [25] (Fig.3H). Strikingly, also AURKA was identified in this assay as being selectively biotinylated, suggesting a novel direct interaction of this kinase and the fusion protein (Fig.3H).

Taken together, our results indicate that AURKA inhibition can decrease fusion protein stability through reduced phosphorylation of serine 437 which might contribute to its ability to induce apoptosis in FP-RMS cells.

Alisertib and ABT-263 act synergistically in vitro

Since it is unlikely that single agents will be able to provide significant clinical benefit, we next aimed to investigate whether a combination of synergistically acting drugs could be identified. To do this in an unbiased way, we screened our library for compounds that would synergistically reduce cell viability in conjunction with a non-effective concentration of ABT-263 (IC_{20}) (Suppl.Fig.5A-B). We assessed cell viability after 48h of combination treatment and ranked the results according to synergistic reduction in viability. Strikingly, out of the 28 hits identified, seven were AURKA inhibitors (Fig.4A). Six of these seven AURKA inhibitors were also identified in a similar screen carried out in a second FP-RMS cell line (Suppl.Fig.5C-D). Hence, AURKA inhibitor might act in synergy with ABT-263. To test this, we selected Alisertib and treated FP-RMS cells with increasing concentrations of both Alisertib and ABT-263 to obtain a combination matrix. Indeed, both drugs in combination were able to induce cell death even at low concentrations (Fig.4B). This combination was highly synergistic as assessed by the SynergyFinder web tool [26] (Fig.4C). Importantly, we observed comparable synergistic effects not only in cell lines but also using cells from patient-derived xenografts (PDX) (Fig.4D-E). This synergy was tumor specific as non-tumorigenic cells (human foreskin fibroblasts and myoblasts) were not sensitive towards the combination treatment (Suppl.Fig.6A-D), whereas we observed a less pronounced synergy in the FN-RMS cell line RD (Suppl.Fig.6E-F).

As AURKA is plays an important role for cell cycle progression during G_2/M phase, we also were interested to assess the effects of Alisertib alone or in combination on cell cycle in FP-RMS cells by flow cytometry. When we analyzed cells treated with 50 or 100 nM Alisertib, we observed an increasing proportion of cells arrested in G_2/M phase (Fig.4F). Additional treatment with 800nM ABT-263, however strongly reduced the G_2/M peak and increased the sub-G_1 fraction (Fig.4F, Suppl.Fig.6G). This suggests that Alisertib induces a cell cycle arrest in G_2/M while in combination with ABT-263 engages apoptosis. These findings are also supported by a synergistic increase in caspase 3/7 activity (Suppl.Fig.6H).
Taken together, our data indicate that AURKA inhibitors act synergistically in combination with ABT-263 in vitro as observed not only in cell lines but also in cells from PDXs, while the drug combination had no major effects in non-tumorigenic cells.

Combination of Alisertib and ABT-263 synergistically reduces tumor growth in vivo

Having confirmed a synergistic action of Alisertib and ABT-263 in vitro, we next aimed to assess an anti-tumorigenic response to the drug combination in vivo. We injected NOD/Scid il2rg$^{-/-}$ (NSG) mice subcutaneously with either Rh4 or patient-derived IC_pPDX35 cells. After tumors were palpable, mice were randomized into four groups and treated daily over three weeks with either vehicle, ABT-263 alone (30 mg/kg), Alisertib alone (80 mg/kg), or combination of both drugs (Suppl.Fig7A). While continuous tumor growth was observed in Rh4 cells in vehicle and ABT-263 only treated mice, Alisertib treatment slightly delayed tumor growth but failed to induce lasting effects (Fig.5A). In contrast, combination therapy resulted in slight tumor regression and lasting stable disease even after the treatment period (Fig.5A). This reduction in tumor growth was also reflected in the survival of mice, where only animals in the combination group survived (Fig.5B). In the PDX, Alisertib treatment alone provoked a stronger delay in tumor growth whereas also in this setting, combination treatment showed the most stable growth control (Fig.5C) and survival was still significantly increased in combination treated mice (Fig.5D). We also isolated tumors after one week of treatment. Histological analysis after one week of treatment revealed a markedly increased number of apoptotic cells in combination treated tumors (Fig.5E). which was also reflected by increased staining for cleaved caspase 3 (Fig.5E). Consequently, proliferation in combination treated tumors was reduced as reflected by reduced signals of Ki67 and increased expression of p21 (Suppl.Fig.7D-E). Furthermore, increased effector caspase cleavage was also found in Western Blot analysis of lysates from tumor tissue (Fig.6F) which showed downregulation of PAX3-FOXO1 (Fig.6F).

Finally, to underscore the clinical relevance of AURKA inhibition, we noticed that AURKA expression is significantly higher in three independent rhabdomyosarcoma datasets compared to biopsies from healthy skeletal muscle (Fig.5G) (data from R2 database: Genomics Analysis and Visualization Platform: *http:///r2.amc.nl*).

Discussion

Our aim for this study was to characterize cell death in alveolar rhabdomyosarcoma (aRMS) following silencing of PAX3-FOXO1 and to translate this knowledge into novel combination therapy approaches. Here, we show that aRMS cells undergo intrinsic apoptosis in a NOXA-dependent manner upon loss of PAX3-FOXO1 expression. In accordance with these findings, we show that BH3-mimetics, especially ABT-263 (Navitoclax), can efficiently facilitate this mode of cell death. Furthermore, we demonstrate a novel functional interaction between Aurora kinase A and the fusion protein. Consequently, we observe strong synergy in vitro and in vivo between the Aurora kinase A inhibitor Alisertib and the BH3-mimetic ABT-263.

We could establish a functional link between the PAX3-FOXO1 and the BH3-only protein NOXA. However, we were unable to show exactly how the fusion protein regulates NOXA expression. In ChIP experiments, we could not find binding of PAX3-FOXO1 to the genomic NOXA locus (unpublished), excluding a direct role of the fusion protein in NOXA regulation. NOXA is well described as a target of p53 signaling [27], but as most other cell lines, the ones we used for our experiments are p53 deficient [28]. However, we observe MYCN-dependent regulation of NOXA expression, in accordance with what has been described recently [29]. While MYCN is known target gene of PAX3-FOXO1 [30], this does not sufficiently explain NOXA upregulation upon silencing of the fusion protein, indicating that there might still be another regulatory pathway involved.

Conversely, we found that combination of Navitoclax with Alisertib reduced NOXA proteins levels after one week of treatment in vivo. This finding indicates that although PAX3-FOXO1 is destabilized, further mechanisms, independent of NOXA contribute significantly to the synergistic effect of the combination. For instance, Aurora kinase A is known to stabilize MYCN [31]. As described above, we found that NOXA expression in aRMS cells is transcriptionally regulated by MYCN (unpublished data). Consequently, Aurora kinase inhibition would reduce MYCN activity and thereby also reduce NOXA expression. This mechanism of action seems to outweigh the upregulation of NOXA after silencing of PAX3-FOXO1.

We found that phosphorylation of PAX3-FOXO1 at serine 437 is important for fusion protein stability. This site corresponds to serine 256 in wild-type FOXO1. Upon phosphorylation of this residue, FOXO1 is excluded from the nucleus and subsequently degraded [32]. Strikingly, phosphorylation of S256 in wild-type FOXO1 and S437 in PAX3-FOXO1 respectively, seem to have opposite effects on protein stability. A similar effect has already been described for acetylation of lysines 426 and 429 that increases stability in PAX3-FOXO1 [33]. Matsuzaki and colleagues could show that acetylation of these corresponding sites in wild-type FOXO1 results in subsequent phosphorylation at S256 [34]. Considering that a similar mechanism is involved in the fusion protein, we can now assume how K426/K429 acetylation contributes to stability: through priming the fusion protein for S437 phosphorylation.

Taken together, we found a previously undescribed site in PAX3-FOXO1 that is important for fusion protein stability. With Aurora kinase A inhibition, we found a therapeutic option to target this site. Furthermore, characterization of the exact cell death mechanism allowed us to find drugs enhancing this mode of cell death. When used in combination, both drugs show a high degree of synergy in vitro and in vivo. These findings shed more light on a devastating disease and may offer novel therapeutic options in the treatment of alveolar rhabdomyosarcoma.

Material & Methods

Cell lines

Rhabdomyosarcoma cell lines RD, Rh4 (both received from Peter Houghton, St. Jude Children's Hospital, Memphis, TN), RhJT (Scott Diede, Fred Hutchinson Cancer Research Center, Seattle, WA), RMS (Janet Shipley, Sarcoma Molecular Pathology, The Institute of Cancer Research, London, UK), KFR (Jindrich Cinatl, Frankfurter Stiftung für krebskranke Kinder, Frankfurt, Germany), Rh30 as well as HEK293T cells (both ATCC LGC Promochem) were cultured in high glucose DMEM (Sigma-Aldrich), supplemented with 100 U/mL penicillin/streptomycin, 2 mmol/L L-glutamine, and 10% FBS (Life Technologies) at 37°C and 5% CO_2 .

Cells from patient-derived xenografts (PDX)

PDX tumors were dissociated as described before [35]. In brief, tumor tissue was minced with scalpels under sterile conditions and suspended in Hanks' Balanced Salt Solution (HBSS, Sigma-Aldrich) supplemented with 1 mmol/L $MgCl_2$, 200 μg/mL Liberase, and 200 U/mL DNase I (both Roche). Tissue

was digested for 30 minutes at 37°C and filtered twice through 70 μm cell strainers (BD Biosciences). Dissociated cells were washed with phosphate-buffered saline (PBS, Sigma-Aldrich) before freezing or resuspending for further culture.

Cells derived from PDX tumors were cultured in Neurobasal medium (Life Technologies) supplemented with 2x B-27™ Supplement (Life Technologies), 20 ng/mL EGF and 20 ng/mL basic FGF (Peprotech) on plates coated with Matrigel® (Corning Life Sciences).

Virus production and transduction

Lentiviral particles (for stable integration of shRNA or CRISPR/Cas9 constructs) were produced in HEK293T cells. In brief, cells were transfected with Pax2 (Addgene #12259) and VSV-G plasmids (Addgene #12259) as well as with the respective transfer plasmid using calcium phosphate transfection. Virus containing supernatant was concentrated using Amicon Ultra centrifugal filter units (Merck Millipore). aRMS cell lines were transduced with the virus particles in presence of hexadimethrine bromide (Sigma-Aldrich). Transduced cells were selected with puromycin (shRNA constructs) or sorted by flow cytometry for expression of blue fluorescent protein (BFP) (CRISPR constructs). pLentiCRISPR-tagBFP (Addgene #75160) was a gift from Beat Bornhauser, Zurich.

In vitro drugs treatments / drug library screenings

Cells were seeded in the respective plate format needed for downstream analysis (i.e. 384-well plate for viability or caspase 3/7 assays). shRNA was induced by diluting doxycycline (Sigma-Aldrich) into the culture medium at a final concentration of 100 ng/mL. Drugs were added to the cells using the HP D300 Digital Dispenser (Tecan) and the corresponding software.

Drug library (Supplemental table 1) was purchased from Selleckchem as pre-dissolved stocks of 10 mmol/L and was stored at -80°C. 24h after seeding cells, medium was changed to 19 μL culture medium (for experiment in Fig. 2 +/- 100 ng/ml doxycycline / for experiment in Fig. 4 +/- 800 nmol/L ABT-263). Library was thawed and pre-diluted to 10 μmol/L in culture medium. From pre-dilution, 1 μL of each drug was added to the wells for a final concentration of 500 nmol/L. Cells were incubated for 48h and viability was measured by WST-1 assay. For results in Fig. 2 in each condition (Rh4 shsc - dox, Rh4 shsc + dox, Rh4 shP3F - dox, Rh4 shP3F + dox) relative cell viability compared to DMSO treatment (= 100%) was calculated for each drug. Relative viability effect was calculated as the ratio between relative viability +dox vs –dox.

For protein screen in Fig. 3, cells were seeded in 24-well plates. 24h later medium was changed to 495 μL culture medium. Library was pre-diluted as described above and 5 μL of pre-dilution was added to each well to reach a final concentration of 100 nmol/L. Cells were prepared for Immunoblot analysis after 48h of incubation.

Antibodies

Antibodies are listed in the Supplemental Material and Methods section.

Viability and caspase assays

WST-1 assay (Roche Diagnostics) to assess cell viability was used according to manufacturer's protocol. In brief, Assay solution was diluted 1:2 in culture medium and added directly into the wells. Cells were incubated at 37°C / 5% CO_2 for 30 minutes and absorption of 440nm was measured against 640nm.

Caspase activity was assessed using Caspase-Glo® 3/7 Assay (Promega) according to manufacturer's protocol. In brief, Assay solution was added directly to each well, plate was covered and shaken, followed by incubation at room temperature for 45 minutes. Signal was assessed by measuring chemiluminescence.

Mouse xenograft experiments

NOD/Scid il2rg$^{-/-}$ (NSG) mice were 8-12 weeks old for the experiments. 5x10^6 Rh4 cells or IC_PPDX35 cells, respectively, were injected subcutaneously into the flanks NSG mice and allowed to engraft. After engraftment mice were randomized into 4 groups (5-6 mice per group) for a group mean in tumor size of 100 mm^3. The groups were treated with vehicle, Alisertib alone, ABT-263 alone, or the combination of both drugs, respectively, according to the regimen shown in Supplemental Figure 7A. Tumor growth was assessed by caliper measurements and the volumes were calculated using the formula

$V= (4/3)\ \pi\ r3$; $r=(d1+d2)/4$). Mice were sacrificed when the tumor volume reached 1000 mm³. All animal experiments have been approved by the Swiss veterinary authorities and were performed according to the animal license ZH206/15

Expression analysis using the R2 database

All datasets are available on the open access platform R2 Genomics and Visualization Platform (http://r2.amc.nl).

Statistics

Data analysis was performed with GraphPad Prism 7. Significance was calculated using unpaired two-tailed Student's t-test or Welch's two-tailed test. Two-way ANOVA was used for multiple comparisons. Differences were considered statistically significant with $p<0.05$. Drug synergy was calculated using the Bliss independence model in the free SynergyFinder WebApp [26].

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Figure legends

Figure 1: Silencing of PAX3-FOXO1 induces apoptosis via NOXA

A. Caspase activity after silencing of PAX3-FOXO1. Caspase 3/7 activity was assessed 24, 48 and 72 hours after induction of shRNA expression. Mean of three independent experiments; bars, SD; 2-way ANOVA, ***, P ≤ 0.001. B. Western blot analysis of whole cell lysates from Rh4 shsc or shP3F 48h after shRNA induction with doxycycline (+) or no induction (-). C. z-vad mediated rescue from cell death. WST-1 assay of Rh4 shsc or shP3F treated with doxycycline as before and additionally treated with increasing concentrations of z-vad FMK. Mean of two independent experiments; bars, SD; Student's *t* test, *, P ≤ 0.05, **, P ≤ 0.01. D. Rescue experiment after shRNA-mediated silencing of PAX3-FOXO1 mRNA. Rh4 cells expressing either scrambled shRNA (shsc) or shRNA targeting PAX3-FOXO1 mRNA (shP3F) were treated for 48h with 0.1 µg/mL doxycycline to induce shRNA expression. Viability was assessed using WST-1 assay and shown relative to non-treated cells. Additionally, cells were treated with either DMSO or a cell death inhibitor at the given concentration. Mean of two independent experiments; bars, SD. E. BH-3 only rescue screen. Caspase 3/7 activity of Rh4 shP3F cells harboring CRISPR/Cas9-induced knockouts of the indicated genes (sc = scrambled sgRNA). White bars, no shRNA induction; black bars, 48h after doxycycline-induced shRNA expression. Mean of two independent experiments; bars, SD. F. NOXA expression after PAX3-FOXO1 silencing. Western blot analysis of whole cell lysates from Rh4 shsc or shP3F 48h after shRNA induction with doxycycline (+) or no induction (-). G. Relative mRNA expression of the PMAIP1 gene in Rh4 shsc or shP3F 48h after induction of shRNA with doxycycline. Gene expression was normalized to GAPDH. Mean of two independent experiments.

Each experiment was performed in triplicates.

Figure 2: ABT-263 sensitizes cell to cell death after silencing of PAX3-FOXO1

A. Drug screen to enhance cell death after silencing of PAX3-FOXO1. Rh4 shsc and Rh4 shP3F cells were treated with drugs from a library (Supplemental table 1) while simultaneously inducing shRNA expression with 0.1 µg/mL doxycycline for 48h. Viability was measured by WST-1 assay and the relative viability effect of each drug was calculated (see Method section). Plot shows the mean viability effects from 3 independent experiments performed in duplicates. B. List of the top hits according to the ratio of relative viability effects of drugs on shP3F over shsc. Ranked according to their targeting class. SD = standard deviation; Welch's two-tailed t-test, *, P ≤ 0.05, **, P ≤ 0.01, ***, P ≤ 0.001. C. Individual effect

of ABT-263 on relative cell viability of Rh4 shsc or Rh4 shP3Fcompared to DMSO; SD; Welch's two-tailed t-test, **, P ≤ 0.01. D-F. Relative cell viability of Rh4 shsc or Rh4 shP3F cells after 48h with or without shRNA induction and simultaneous treatment with increasing concentrations of ABT-263 or ABT-199, respectively. Mean +/- SD from three independent experiments performed in triplicates. IC_{50} values were calculated from non-linear regression analysis using GraphPad Prism® 7. G. Relative cell viability of Rh4 shsc *PMAIP*$^{-/-}$ or Rh4 shP3F *PMAIP*$^{-/-}$ cells treated with increasing concentrations of ABT-263 for 48h.

Figure 3: Inhibition of Aurora Kinase A leads to reduced PAX3-FOXO1 protein stability

A. PAX3-FOXO1 protein levels were assessed by Western Blot 48h after treatment with 100 nmol/L of each compound of a drug library (Supplemental table 1). Protein bands were analyzed by densitometry and normalized to GAPDH levels. Treatment effect was compared to DMSO treatment. B. Exemplary blot of one set of drugs. Aurora Kinase A inhibitors are labelled in red. C: Chart showing the classes of inhibitors found among the top candidates. Red: Aurora Kinase A inhibitor, green: CDK9 inhibitors, grey: epigenetic modulators. D. Western Blot of lysates from Rh4 cells treated for 48h with increasing concentrations of the given drug. Left panel: Alisertib, right panel: AT9283. E. Immunoblot for phosphorylation at the given sites. Cells were incubated for 24h with Alisertib and lysed with Co-IP buffer. F. Western Blot of RD cells transiently transfected with the respective overexpression plasmid for PAX3-FOXO1 mutants or wild-type (wt). 8h before lysis cells were treated with either 10 µg/mL cycloheximide (CHX) or DMSO to block protein synthesis. G. Densitometric quantification of the fusion protein levels, normalized to GAPDH. Mean of three independent experiments; bars, SD; two-way ANOVA, *, P < 0.05, **, P < 0.01. H. Western Blot analysis of streptavidin pull-down experiments. HEK293T cells were transduced with a PAX3-FOXO1 expression plasmid fused to BirA biotin ligase (P3F-BirA) or with GFP or BirA alone. Cells were incubated with biotin (+) or not treated (-) and lysed. Pull-down was performed with beads coated with streptavidin.

Figure 4: ABT-263 and Alisertib synergistically induce cell death in vitro

A. Synergy screen. Rh4 cells were incubated with each drug of a library (Supplemental table 1) at a concentration of 500 nmol/L and additionally with either 800 nmol/L ABT-263 or DMSO. After 48h viability was assessed by WST-1 assay. Left y-axis: black bars, compound; grey bars, compound + ABT-263. Right y-axis, red bars: viability ratio (+ABT-263/+DMSO). Top hits with a viability ratio < 0.7

are shown and ranked according to viability of each drug alone. Red stars: Aurora Kinase A inhibitors. Circle plot: 28 top hits were classified according to their target spectrum: red, Aurora Kinase A inhibitors. B. Relative cell viability in % after cross-titration of Alisertib against ABT-263 in Rh4 cells. Viability relative to DMSO control after 48h. Color scheme: high viability, blue; low viability: red. Mean values of three independent experiments performed in duplicates. C. 3D representation of synergy scores of cross-titration experiments. Synergy was calculated according to the Bliss independence model using the SynergyFinder WebApp [26]. Positive values (red) indicate synergy, negative values (green) indicate antagonism. D. Cross-titration of Alisertib against ABT-263 in IC_PPDX35-derived PDX cells. Mean values of three independent experiments performed in duplicates.E. Synergy scores of IC_PPDX35 cross-titration. F. Rh4 cells were treated with given concentrations of Alisertib and additionally with either DMSO or 800 nmol/L ABT-263. After 24h cells were stained with propidium iodide (PI) and cell cycle was analyzed by flow cytometry. Mean of two independent experiments.

Figure 5: Combination of ABT-263 and Alisertib reduces tumor growth in vivo

Rh4 or IC_pPDX35 cells were injected s.c. into the flanks of NSG mice. After engraftment, mice were randomized and assigned into one of four treatment groups: Vehicle (black), ABT-263 only (grey, 3d / week 80 mg/kg), Alisertib only (beige, 5d / week 30 mg/kg), ABT-263 + Alisertib combination (red). Mice were treated for 3 weeks with the respective regimen through administration p.o. (see also Suppl.Fig.7A) and sacrificed when tumors reached a size of 1000 mm^3.

A. Tumor growth of Rh4 cells in vivo. Black arrow: start of treatment. Red box indicates treatment period. Per group: n = 6; error bars, S.E.M. B. Kaplan-Meier graph showing percent survival of different treatment groups. Mantel-Cox test for comparison of survival curves, ***, p = 0.001 C. Tumor growth of IC_pPDX35 tumors. Black arrow: start of treatment. Red box indicates treatment period. Per group: n = 5; error bars, S.E.M. D. Kaplan-Meier graph showing percent survival. E. Histology of engrafted IC_pPDX35 tumors after one week of treatment with either vehicle control (left panel) or combination of ABT-263 (80 mg/kg) and Alisertib (30 mg/kg) (right panel). N = 3. Upper panel: Hematoxylin & Eosin (HE) staining. Bars = 20 µm. Black arrows: apoptotic cells, red arrow: mitotic cell. Lower panel: Immunohistochemical staining against cleaved caspase 3. Bars = 50 µm. Representative images of sections from 3 different mice per group. F. Western Blot analysis of protein lysates from IC_pPDX35 tumors after one week of treatment. Mice were treated either with vehicle or the combination of Alisertib (30 mg/kg) and ABT-263 (80 mg/kg) for one week. Mice were sacrificed, and proteins were extracted

from tumor tissues G. AURKA gene expression in rhabdomyosarcoma biopsies and healthy skeletal muscle tissue as determined in different datasets using the r2 database (R2: Genomics Analysis and Visualization Platform: *http://r2.amc.nl*). Violin box-plots, minimum to maximum; unpaired t-test, ****, $p < 0.0001$.

Supplemental figure 1:

A. Schematic of the expression construct showing promoters (pink) and the following ORFs (light blue). hPGK promoter is constitutively active, U6Tet is inducible through tetracycline / doxycycline. B. Relative mRNA expression of the PAX3-FOXO1 gene in Rh4 shsc cells (beige) or Rh4 shP3F cells (red). Gene expression has been normalized to GAPDH and is shown as % of un-induced shRNA (Ctrl); error bars, SD; student's t-test, *** $p < 0.001$. C. Relative mRNA expression of PAX3-FOXO1 gene in the given aRMS cell lines after 48h of shRNA induction with 0.1 µg/mL doxycycline. Beige: shsc, red: shP3F. Expression was normalized to GAPDH and shown relative to un-induced control (Ctrl); error bars, SD, student's t-test, * $p < 0.05$. D. Western Blot analysis of PAX3-FOXO1 (P3F) protein expression in the given cell lines after 48h of shRNA induction (+) or control (-). E. Caspase activity after silencing of PAX3-FOXO1 in Rh30 shsc (beige) and shP3F (red) cells. Caspase 3/7 activity was assessed 24, 48 and 72 hours after induction of shRNA expression. Mean of three independent experiments performed in triplicates; bars, SD. F. Caspase activity in Rh4 cells after silencing of endogenous PAX3-FOXO1 and rescue with over expression of either mutated PAX3-FOXO1 cDNA (silent single base mutations, not-targetable by shRNA, P3F mut, red) or empty vector (pRR, red). Caspase activity was assessed after 48h for three different targeting shRNAs and a scrambled control (sh scr). Error bars, SD; student's t-test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Mean of three independent experiments performed in triplicates. G. Western Blot analysis of rescue experiment.

Supplemental Figure 2:

A. Schematic of the lentiviral CRISPR construct: pink: LTRs and U6-promoter, blue: sgRNA and Cas9 cDNA + EGFP cDNA fused via a P2A self-cleavage site. B-C. Immunoblots to confirm CRISPR-mediated knockout of gene expression in Rh4sh cells. D. BH-3 only rescue screen corresponding to Fig. 1E. Caspase 3/7 activity of Rh4 shsc cells harboring CRISPR/Cas9-induced knockouts of the indicated genes (sc = scrambled sgRNA). White bars, no shRNA induction; black bars, 48h after doxycycline-induced shRNA expression. Mean of two independent experiments; bars, SD. E.

Immunoblot of both Rh4shsc and Rh4shP3F lysates to confirm NOXA knockout. Each cell was tested with scrambled sgRNA (scr) or one of two different sgRNAs targeting the PMAIP1 (NOXA) gene (NOXA1 and NOXA2). In addition, cells were treated for 6h with 10 µmol/L MG-132 to inhibit proteasomal degradation and stabilize potential NOXA proteins. F. Rescue experiment measuring caspase activity in 3 aRMS cell lines 48h after induction of shP3F (black bars) or shsc (white bars). In addition to shRNA cells were transduced with either non-targeting sgRNA (sc) or two different sgRNAs targeting the PMAIP1 (NOXA) gene. Mean of two independent experiments performed in triplicates; bars, SD. G. upper panel: immunoblot for NOXA protein expression; lower panel densitometric quantification of NOXA protein levels normalized to GAPDH and relative to uninduced shRNA (-).

Supplementary Figure 3:

A. Schematic of the drug screen setup. Rh4sh cells were plated in 384-well plates, treated with 500 nmol/L of each drug and 0.1 µg/mL doxycycline to induced shRNA. After 48h WST-1 assay was performed to assess viability. B-M. Individual effect of drugs on relative cell viability of Rh4 shsc or Rh4 shP3F compared to DMSO; SD; Welch's two-tailed t-test, *, P < 0,05; **, P ≤ 0.01. N-O. Relative cell viability of Rh4 shsc or Rh4 shP3F cells after 48h with or without shRNA induction and simultaneous treatment with increasing concentrations of TW-37 or UMI-77, respectively. Mean +/- SD from three independent experiments performed in triplicates. IC_{50} values were calculated from non-linear regression analysis using GraphPad Prism® P. PMAIP1 (NOXA) gene expression in rhabdomyosarcoma biopsies and healthy skeletal muscle tissue as determined in different datasets using the r2 database (R2: Genomics Analysis and Visualization Platform: *http://r2.amc.nl*). Violin box-plots, minimum to maximum; unpaired t-test, ****, p < 0.0001.

Supplemental Figure 4:

A. Screening setup for the library screen to assess PAX3-FOXO1 protein levels. Rh4 cells were plated in 24-well plates and incubated with 100 nmol/L of each drug. Cells were lysed after 48h and processed for western blot analysis. B. Immunoblot performed with individual hits from drug screen to assess dose dependent effects on PAX3-FOXO1 protein levels normalized to GAPDH. C. Immunoblot for MAST60 PDX cells treated for 48h with the given drug and concentration.

Supplemental Figure 5:

A. Relative cell viability of Rh4 and RHJT cells after 48h of treatment with increasing concentrations of ABT-263 (Navitoclax) to determine IC_{20}. Mean of five (Rh4) or two (RHJT) independent experiments performed in triplicates. Bars, SD. IC_{20} values were calculated from non-linear regression analysis using GraphPad Prism® C. Synergy screen. RHJT cells were incubated with each drug of the drug library (Supplemental table 1) at a concentration of 500 nmol/L and additionally with either 250 nmol/L ABT-263 (= IC_{20}) or DMSO. After 48h viability was assessed by WST-1 assay. Left y-axis: black bars, compound; grey bars, compound + ABT-263. Right y-axis, red bars: viability ratio (+ABT-263/+DMSO). Top hits with a viability ratio < 0.7 are shown and ranked according to viability of each drug alone. Red stars: Aurora Kinase A inhibitors. D. Venn diagram with top hits from the synergy screens in both Rh4 (red) and RHJT (blue) cells. Overlap shows common top hits of both cell lines.

Supplemental Figure 6:

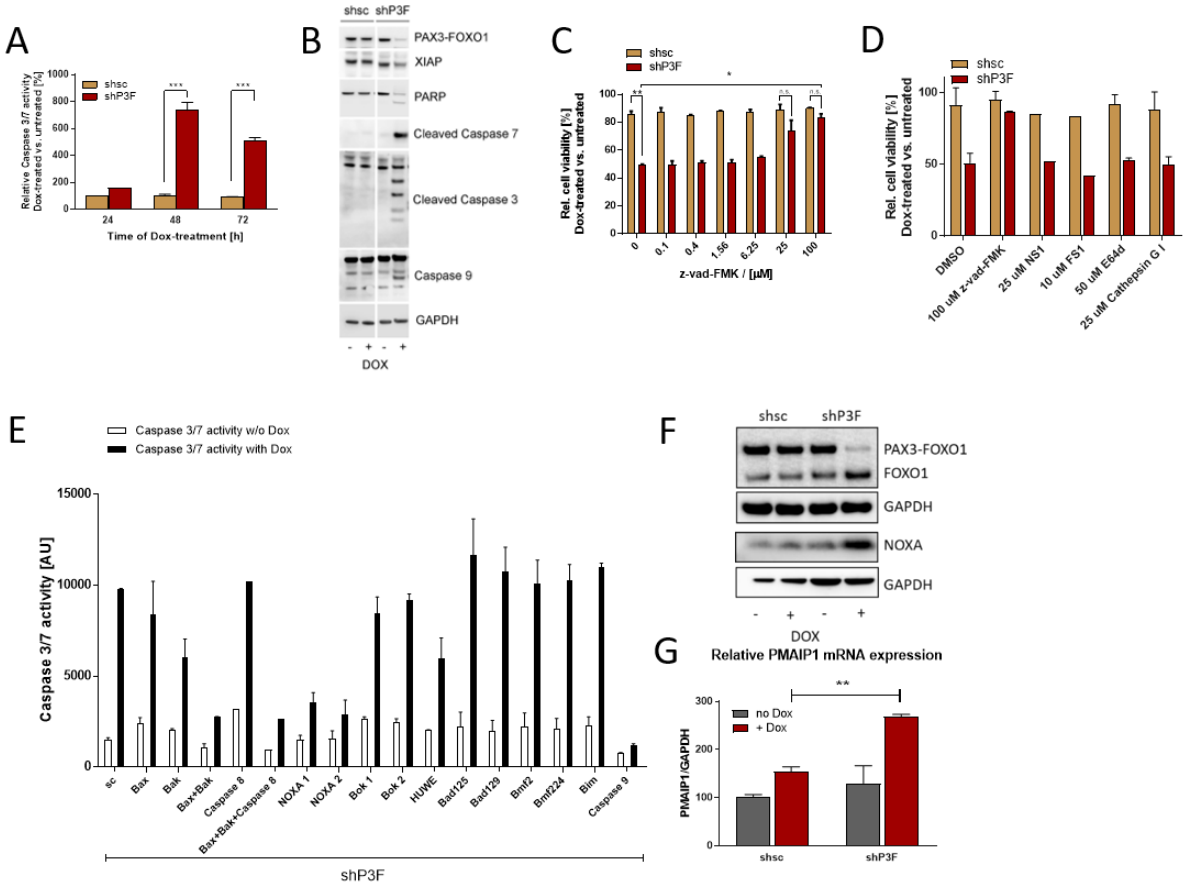
A, C, E. Relative cell viability in % after cross-titration of Alisertib against ABT-263 in myoblasts, human foreskin fibroblasts and RD cells, respectively. Viability relative to DMSO control after 48h. Color scheme: high viability, blue; low viability: red. Mean of two independent experiments performed in duplicates. B, D, F. 3D representations of synergy scores of corresponding cross-titration experiments. Synergy was calculated according to the Bliss independence model using the SynergyFinder WebApp [26]. Positive values (red) indicate synergy, negative values (green) indicate antagonism. G. Brightfield microscopy of Rh4 cells treated with increasing doses of Alisertib and a fixed concentration of 800 nmol/L ABT-263 or DMSO. Representative pictures of three independent experiments. H. Relative caspase 3/7 activity in Rh4 cells after cross-titration of Alisertib against ABT-263 in Rh4 cells after 48h of incubation. Mean of two independent experiments.

Supplemental Figure 7:

A. Experimental setup for in vivo combination treatment. NSG-mice were injected with $5*10^6$ tumor cells (Rh4 or IC_PPDX35). After engraftment mice were randomized into four groups and treated with either vehicle, ABT-263 alone, Alisertib alone, or the combination of both for three weeks. Arrows indicate the days of treatment. At tumor volumes of 1000 mm^3 mice were sacrificed. B-C. Weight curves of mice during the experiment. D-E. Histology of engrafted IC_pPDX35 tumors after one week of treatment with

either vehicle control (left panel) or combination of ABT-263 (80 mg/kg) and Alisertib (30 mg/kg) (right panel). N = 3. D: Ki67. E: p21. Bars = 50 µm. Representative images of sections from 3 different mice per group.

Figure 1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Relative Caspase 3/7 activity Dox-treated vs. untreated [%]
shsc
shP3F
Time of Dox-treatment [h]
B
shsc shP3F
PAX3-FOXO1
XIAP
PARP
Cleaved Caspase 7
Cleaved Caspase 3
Caspase 9
GAPDH
DOX
C
shsc
shP3F
Rel. cell viability [%] Dox-treated vs. untreated
z-vad-FMK / [µM]
D
shsc
shP3F
Rel. cell viability [%] Dox-treated vs. untreated
DMSO
100 uM z-vad-FMK
25 uM NS1
10 uM FS1
50 uM E64d
25 uM Cathepsin G I
E
Caspase 3/7 activity w/o Dox
Caspase 3/7 activity with Dox
Caspase 3/7 activity [AU]
sc
Bax
Bak
Bax+Bak
Caspase 8
Bax+Bak+Caspase 8
NOXA 1
NOXA 2
Bok 1
Bok 2
HUWE
Bad125
Bad129
Bmf2
Bmf224
Bim
Caspase 9
shP3F
F
shsc
shP3F
PAX3-FOXO1
FOXO1
GAPDH
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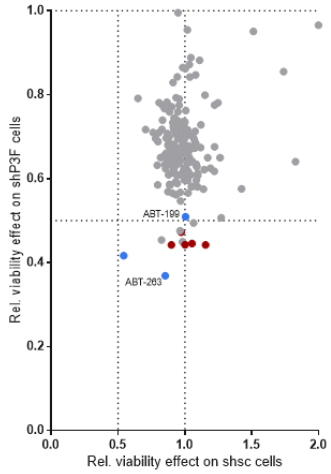
+ Dox
PMAIP1/GAPDH
shsc
shP3F

Figure 2

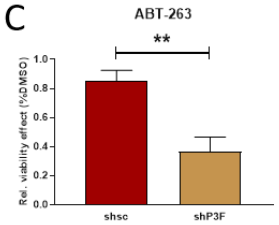
A



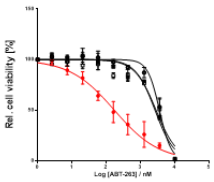
B

Name	Class	Mean viability effect	SD	Mean viability effect	SD	p-value
		Rh4 shsc		Rh4 shP3F		
ABT-263	BH3-mimetic	0.852	0.0606	0.369	0.0799	**
Obatoclax		0.543	0.3474	0.416	0.2277	n.s.
ABT199		1.005	0.0142	0.509	0.0144	**
AT9283	Aurora Kinase A inhibitor	0.899	0.0632	0.442	0.0527	**
Danusertib		1.154	0.2453	0.442	0.0812	*
Tozasertib		1.001	0.1441	0.442	0.0754	*
Alisertib		1.053	0.1868	0.445	0.0623	*
AMG-900		0.972	0.0968	0.473	0.0639	**
Rigosertib	PLK1 inhibitor	0.982	0.0531	0.45	0.2448	n.s.
Dasatinib	Bcr-Abl inhibitor	0.826	0.0221	0.454	0.0271	***
Ispinesib	KSP inhibitor	0.964	0.0785	0.476	0.1778	*
OTX015	Bromodomain inh	1.065	0.2235	0.494	0.0405	*
AZD7762	Chk1 inhibitor	1.271	0.1816	0.507	0.2735	**

C



D ABT-263



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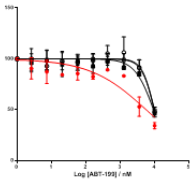
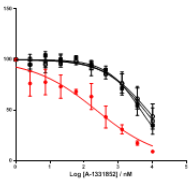
IC50 = 0.18 µM

E ABT-199



IC50 = 9.2 – 10 µM
IC50 = 5.6 µM

F A-1331852



IC50 = 4.5 – 7.0 µM
IC50 = 0.23 µM

G ABT-263

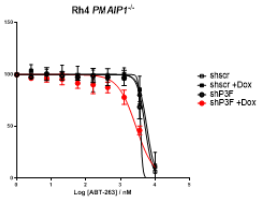
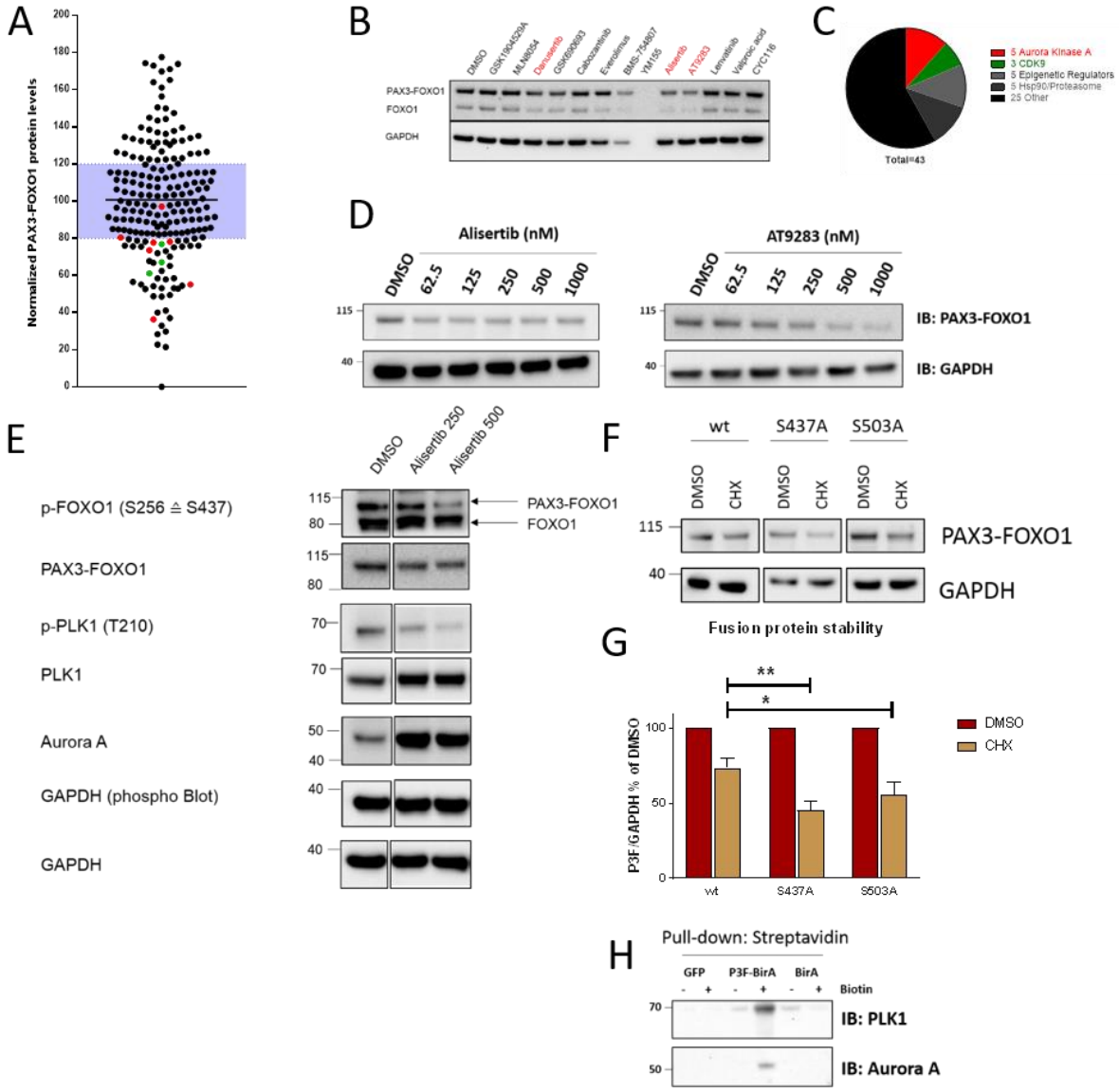


Figure 3


A
Normalized PAX3-FOXO1 protein levels
B
DMSO
GSK1904529A
MLN8054
Danusertib
GSK690693
Cabozantinib
Everolimus
BMS-754807
YM155
Alisertib
AT9283
Linsitinib
Valproic acid
CYC116
PAX3-FOXO1
FOXO1
GAPDH
C
5 Aurora Kinase A
3 CDK9
5 Epigenetic Regulators
5 Hsp90/Proteasome
25 Other
Total=43
D
Alisertib (nM)
AT9283 (nM)
DMSO
62.5
125
250
500
1000
IB: PAX3-FOXO1
IB: GAPDH
E
p-FOXO1 (S256 ≙ S437)
PAX3-FOXO1
p-PLK1 (T210)
PLK1
Aurora A
GAPDH (phospho Blot)
GAPDH
DMSO
Alisertib 250
Alisertib 500
PAX3-FOXO1
FOXO1
F
wt
S437A
S503A
DMSO
CHX
PAX3-FOXO1
GAPDH
G
Fusion protein stability
P3F/GAPDH % of DMSO
DMSO
CHX
wt
S437A
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Pull-down: Streptavidin
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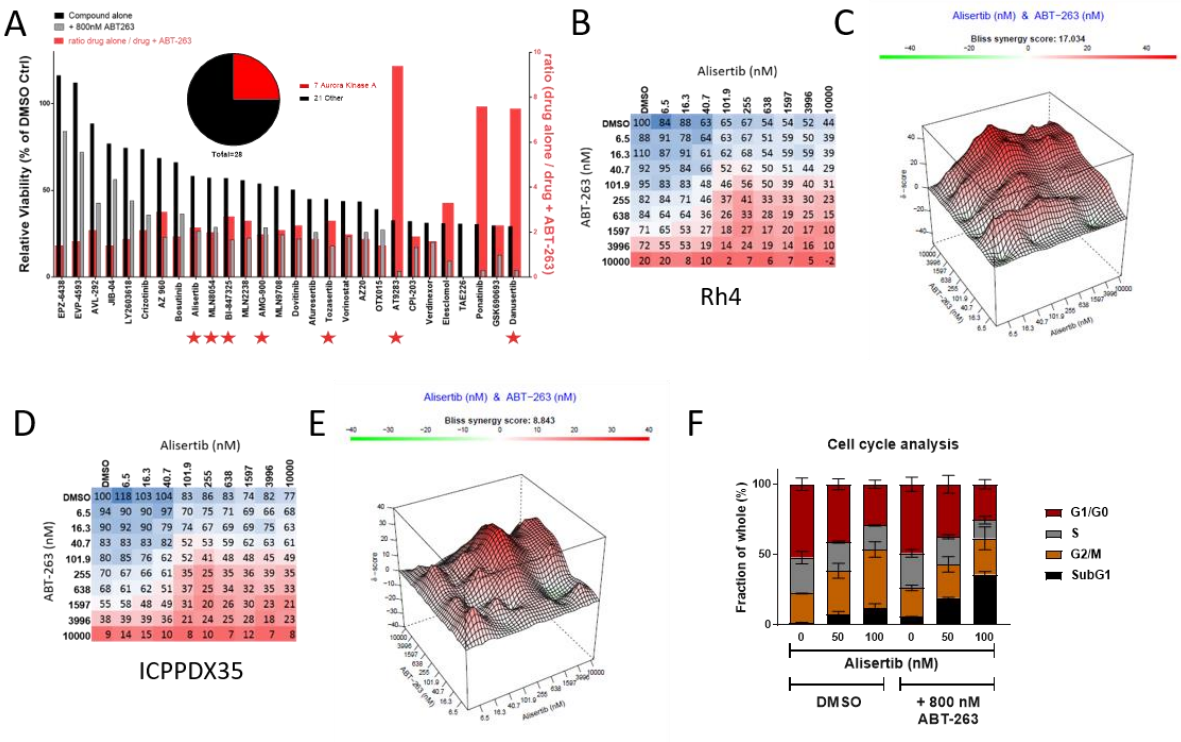
BirA
Biotin
IB: PLK1
IB: Aurora A

Figure 4


A
Compound alone
+ 800nM ABT263
ratio drug alone / drug + ABT-263
Relative Viability (% of DMSO Ctrl)
ratio (drug alone / drug + ABT-263)
7 Aurora Kinase A
21 Other
Total=28
EPZ-6438
EVP-4593
AVL-292
JIB-04
LY2603618
Crizotinib
AZ 960
Beautifolin
Alisertib
MLN8054
BI-847325
MLN8238
AMG-900
MLN9708
Dovitinib
Aflurosertib
Tozasertib
Vorinostat
AZ20
OTX015
AT9283
CPI-203
Verdinexor
Elesclomol
TAE226
Ponatinib
GSK690693
Danusertib
B
Alisertib (nM)
ABT-263 (nM)
DMSO 100 84 88 63 65 67 54 54 52 44
6.5 88 91 78 64 63 67 51 59 50 39
16.3 110 87 91 61 62 68 54 59 59 39
40.7 92 95 84 66 52 62 50 51 44 29
101.9 95 83 83 48 46 56 50 39 40 31
255 82 84 71 46 37 41 33 33 30 23
638 84 64 64 36 26 33 28 19 25 15
1597 71 65 53 27 18 27 17 20 17 10
3996 72 55 53 19 14 24 19 14 16 10
10000 20 20 8 10 2 7 6 7 5 -2
Rh4
C
Alisertib (nM) & ABT−263 (nM)
Bliss synergy score: 17.034
D
Alisertib (nM)
ABT-263 (nM)
DMSO 100 118 103 104 83 86 83 74 82 77
6.5 94 90 90 97 70 75 71 69 66 68
16.3 90 92 90 79 74 67 69 69 75 63
40.7 83 83 83 82 52 53 59 62 63 61
101.9 80 85 76 62 52 41 48 48 45 49
255 70 67 66 61 35 25 35 36 39 35
638 68 61 62 51 37 25 34 32 35 33
1597 55 58 48 49 31 20 26 30 23 21
3996 38 39 39 36 21 24 25 28 18 23
10000 9 14 15 10 8 10 7 12 7 8
ICPPDX35
E
Alisertib (nM) & ABT−263 (nM)
Bliss synergy score: 8.843
F
Cell cycle analysis
Fraction of whole (%)
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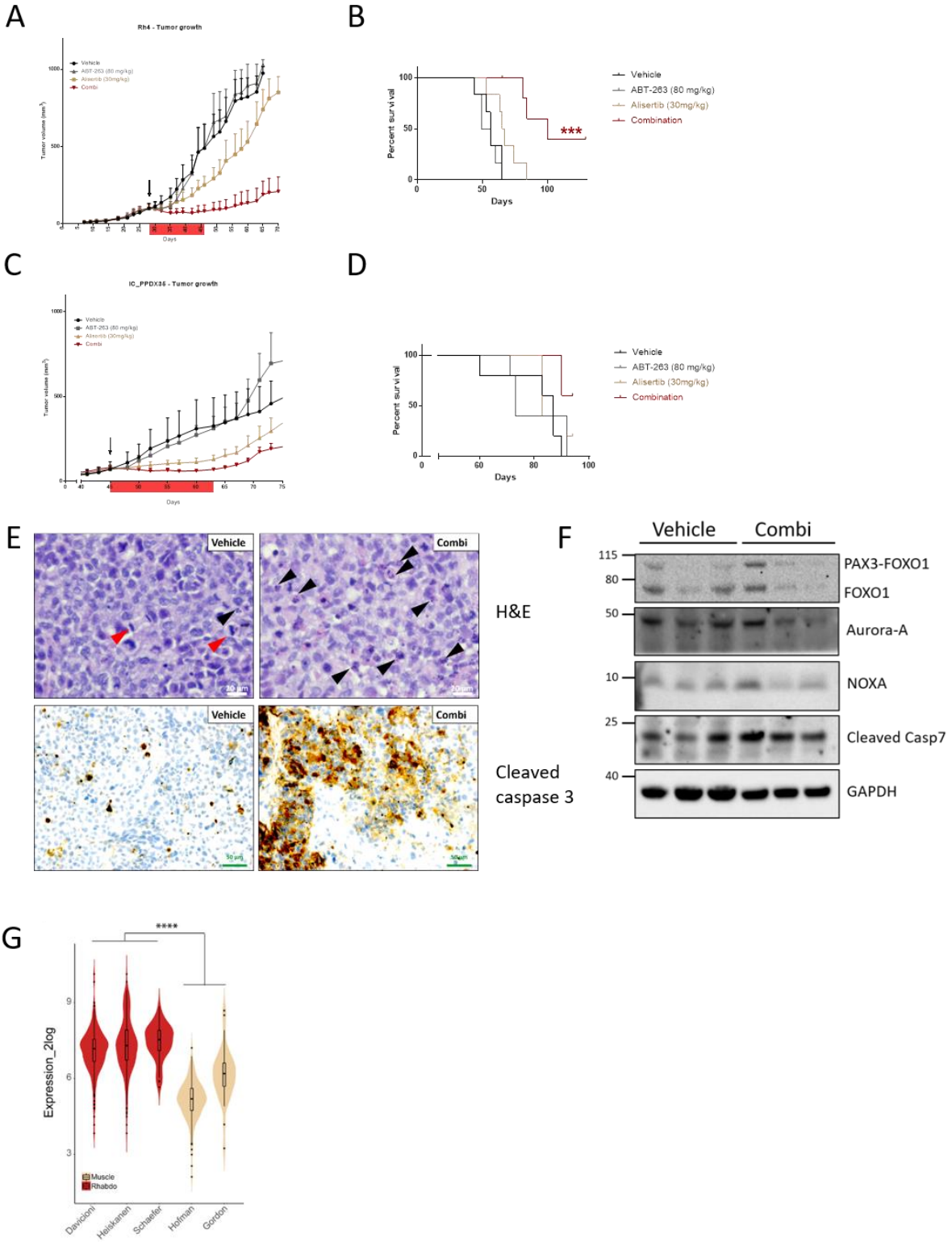
S
G2/M
SubG1
0 50 100 0 50 100
Alisertib (nM)
DMSO
+ 800 nM ABT-263

Figure 5


A
Rh4 - Tumor growth
Vehicle
ABT-263 (80 mg/kg)
Alisertib (30mg/kg)
Combi
Tumor volume (mm³)
Days
B
Percent survival
Days
***
Vehicle
ABT-263 (80 mg/kg)
Alisertib (30mg/kg)
Combination
C
IC_PPDX35 - Tumor growth
Vehicle
ABT-263 (80 mg/kg)
Alisertib (30mg/kg)
Combi
Tumor volume (mm³)
Days
D
Percent survival
Days
Vehicle
ABT-263 (80 mg/kg)
Alisertib (30mg/kg)
Combination
E
Vehicle
Combi
H&E
Vehicle
Combi
Cleaved caspase 3
F
Vehicle
Combi
115
80
50
10
25
40
PAX3-FOXO1
FOXO1
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NOXA
Cleaved Casp7
GAPDH
G
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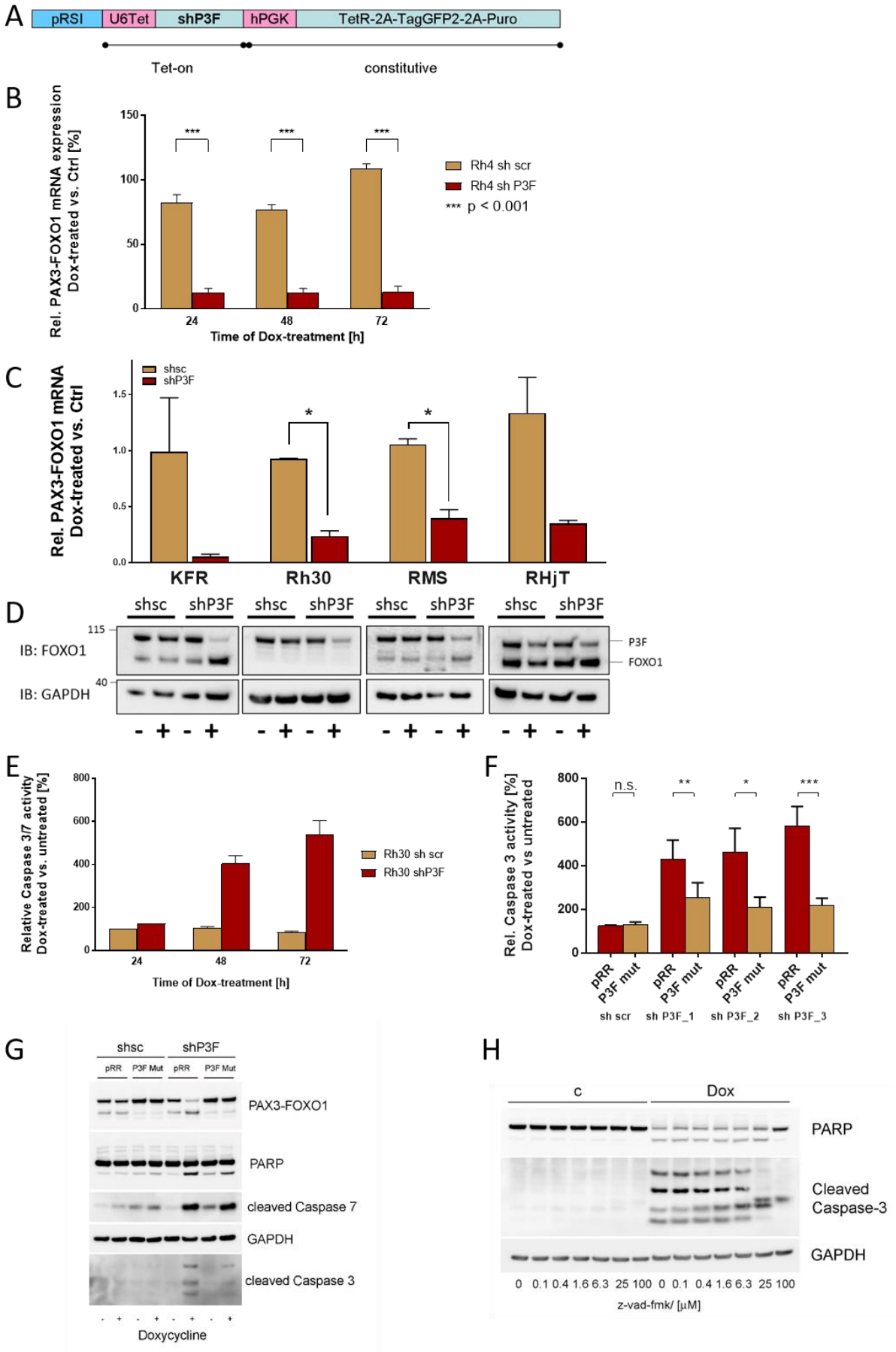
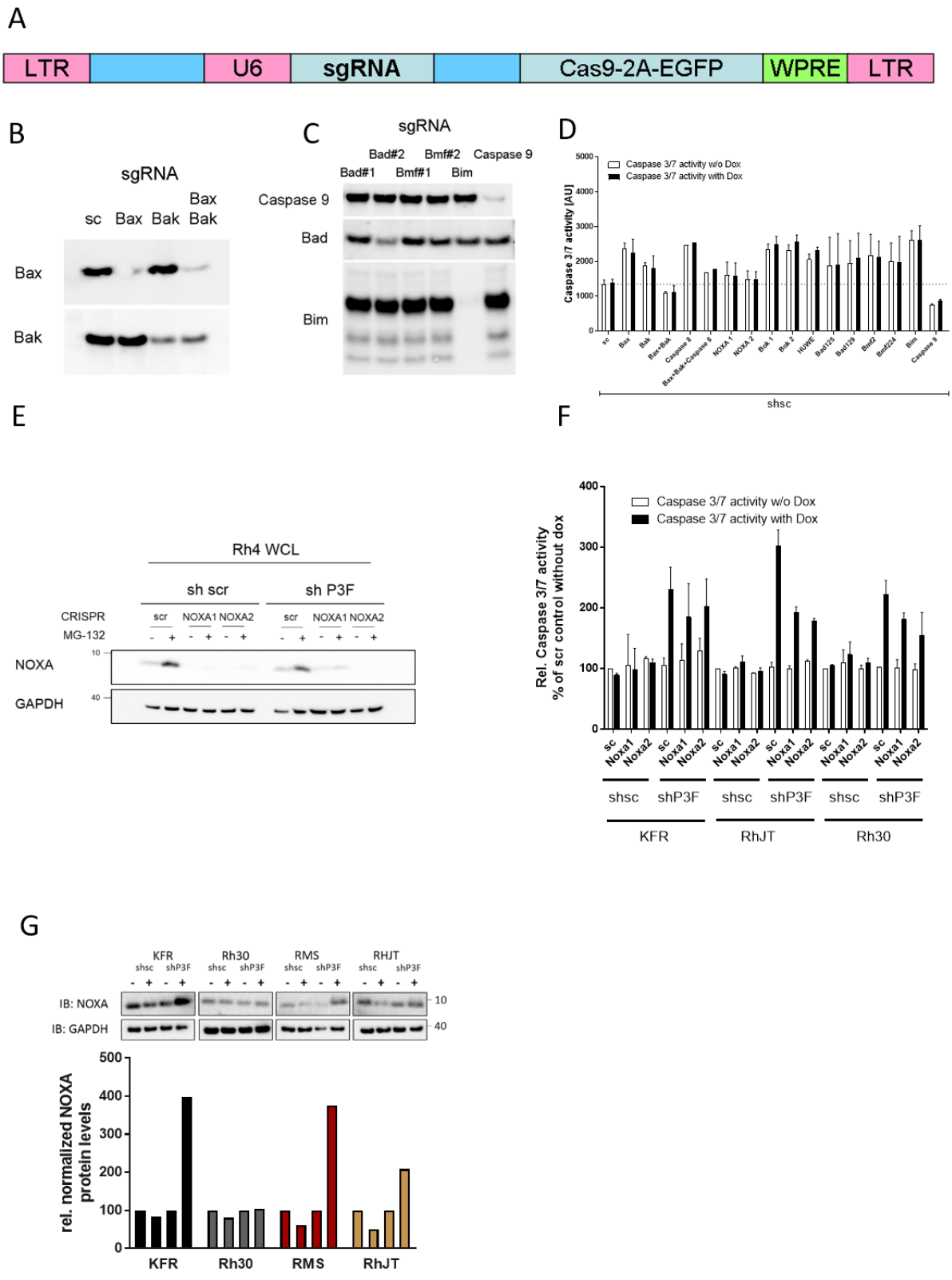
6
3
Muscle
Rhabdo
Davicioni
Heiskanen
Schaefer
Hofman
Gordon

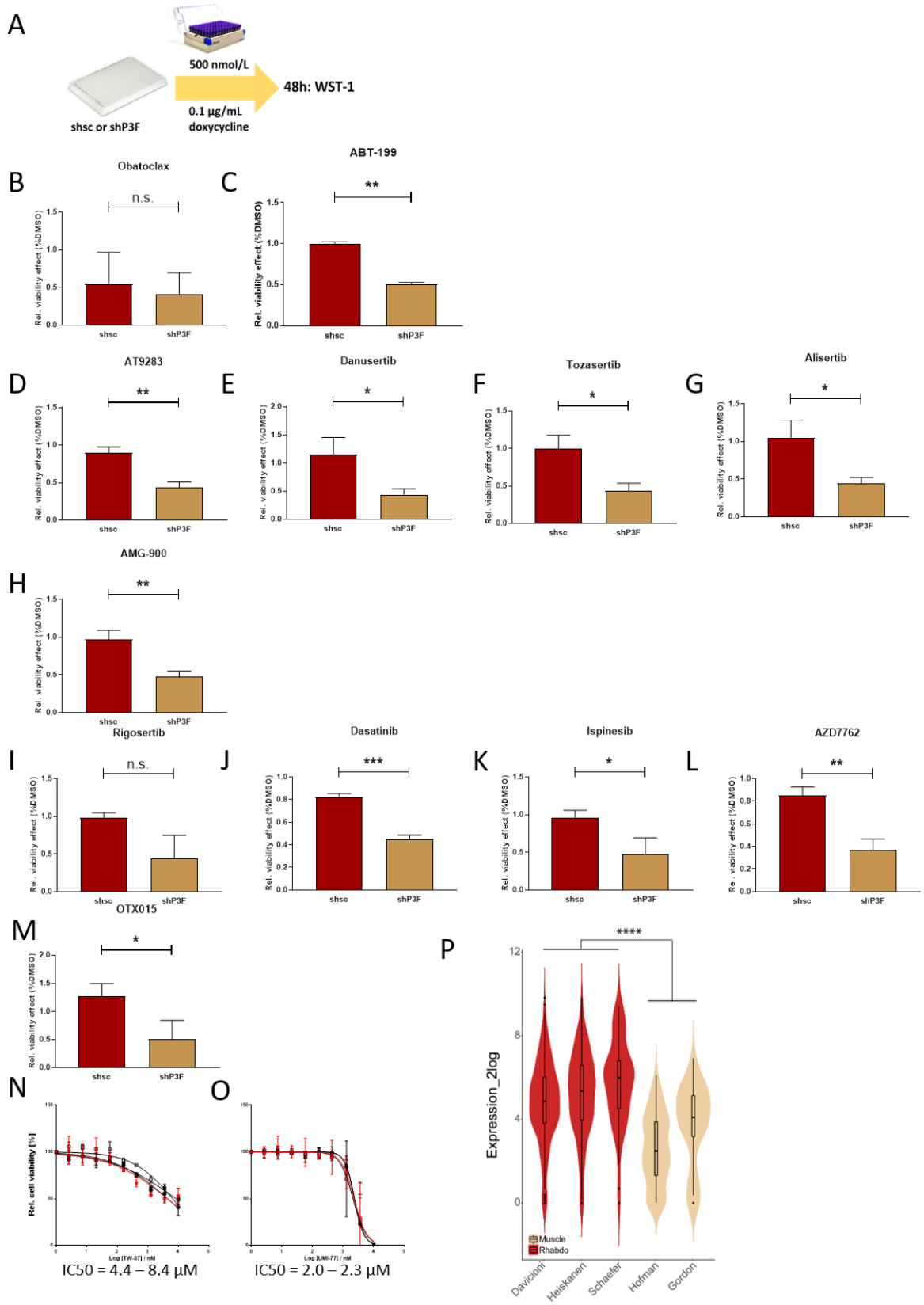
Supplemental Figure 1



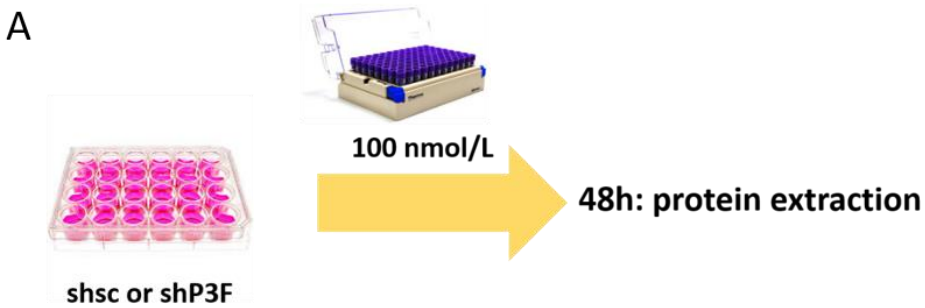
Supplemental Figure 2

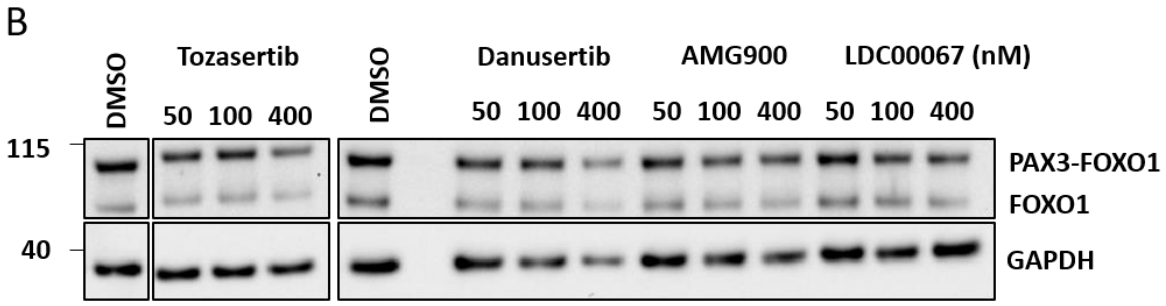


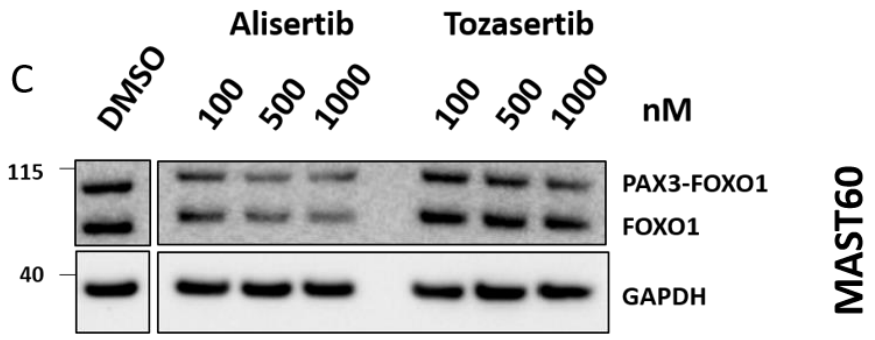
Supplemental Figure 3



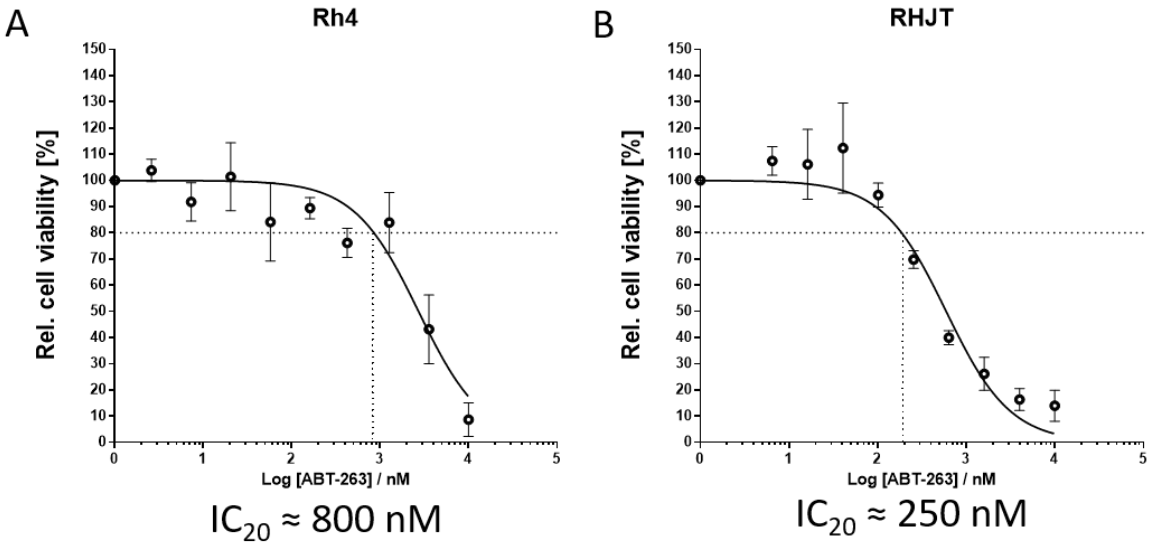
Supplemental Figure 4

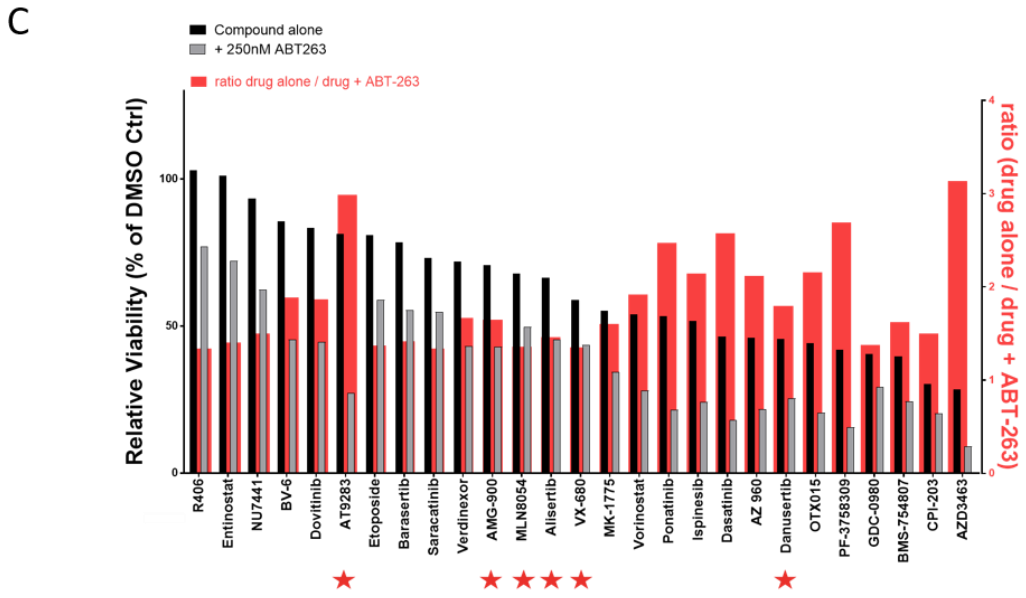


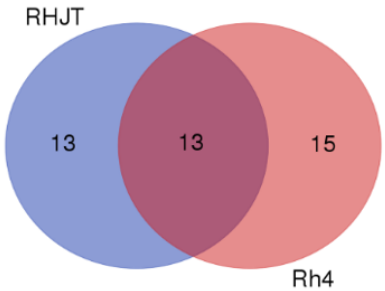




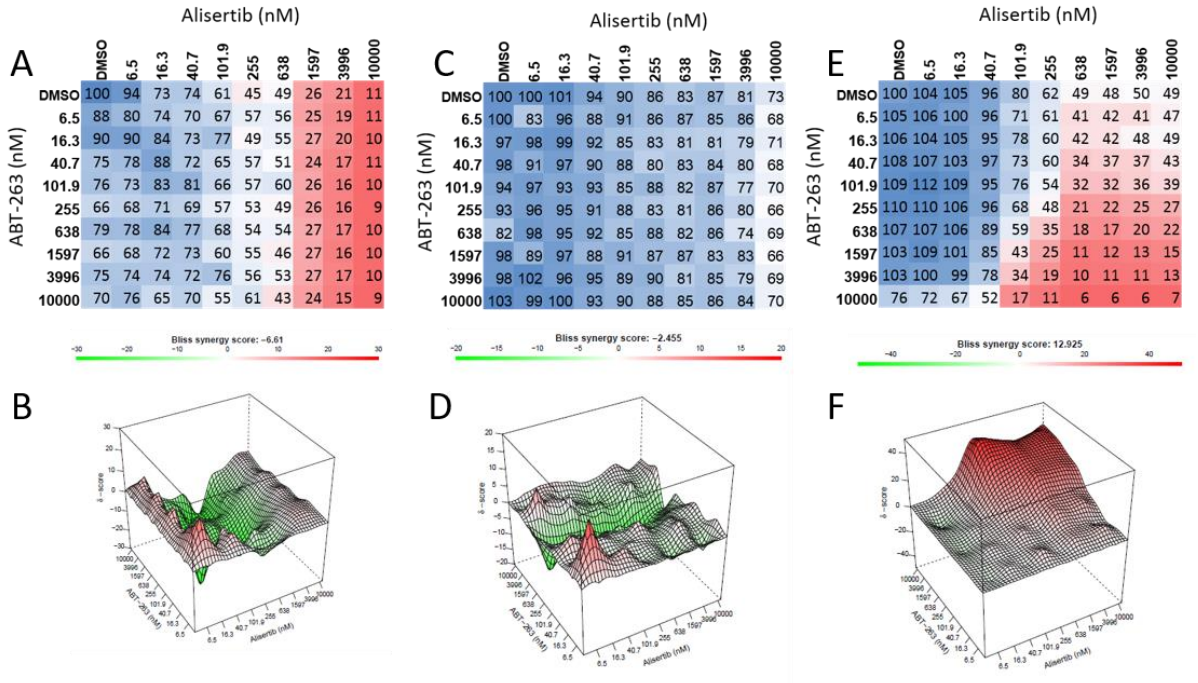
Supplemental Figure 5

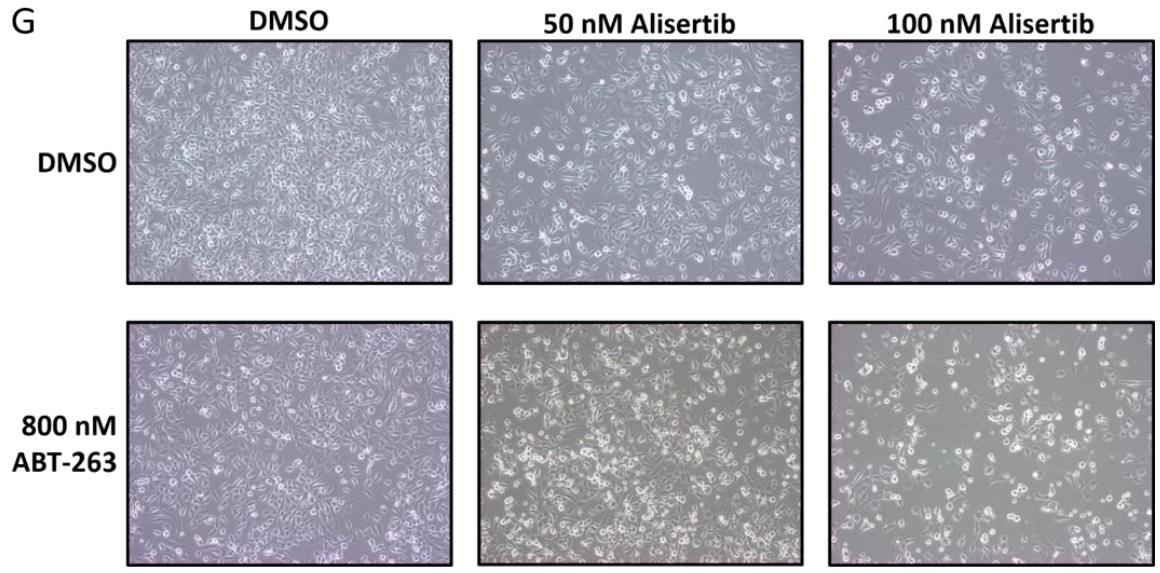






Supplemental Figure 6



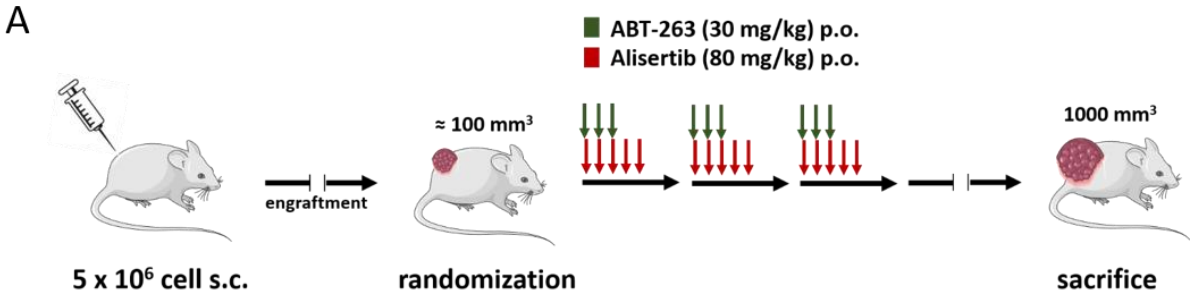


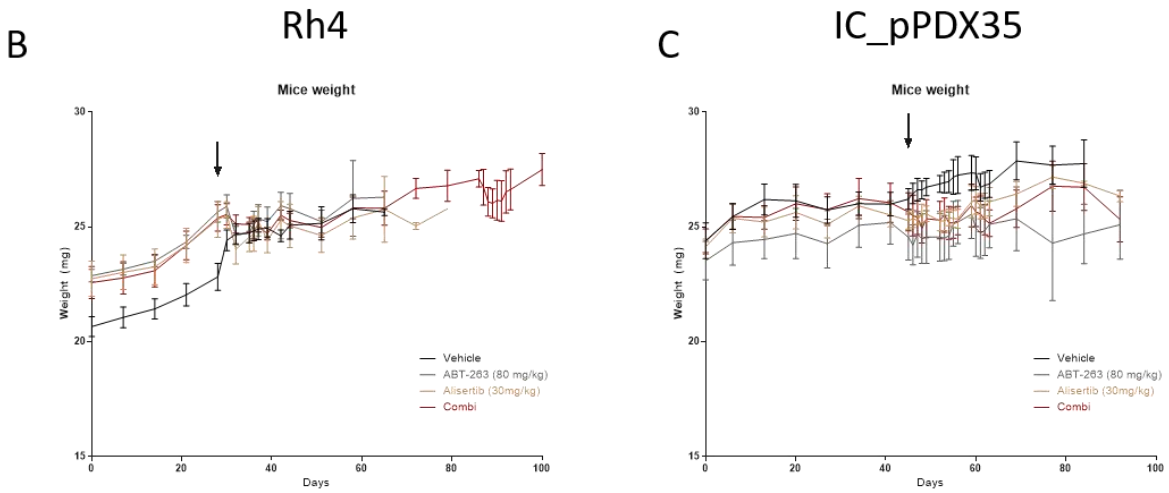
H

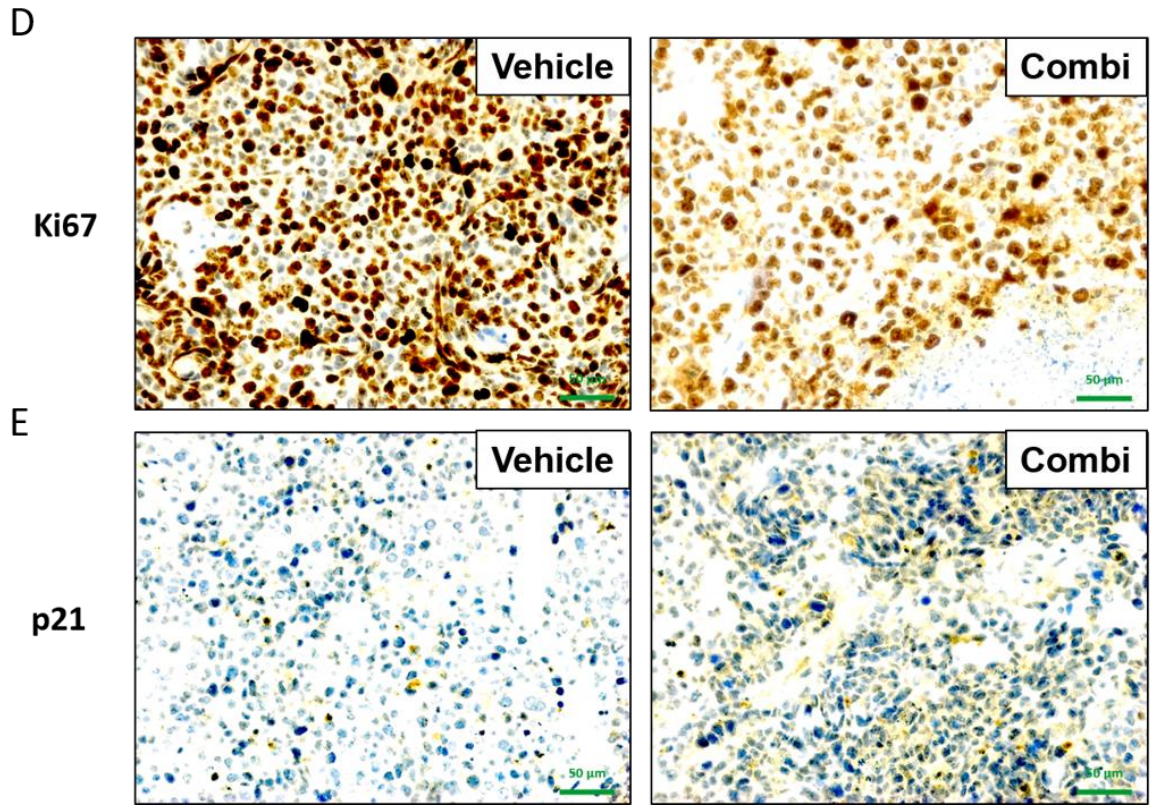
Alisertib (nM)

ABT-263 (nM)	DMSO	6.5	16.3	40.7	101.9	255	638	1597	3996	10000
DMSO	100	94	97	189	194	223	255	295	413	433
6.5	98	117	108	220	205	236	237	308	409	466
16.3	91	107	115	208	234	261	272	311	431	463
40.7	105	137	112	215	281	307	330	356	430	473
101.9	143	145	173	300	367	410	413	451	513	481
255	176	191	162	340	446	509	443	487	524	468
638	209	200	238	477	561	594	508	554	541	383
1597	239	274	304	531	612	611	574	623	511	380
3996	431	370	440	643	681	622	638	578	545	402
10000	823	878	996	884	772	728	676	709	481	420

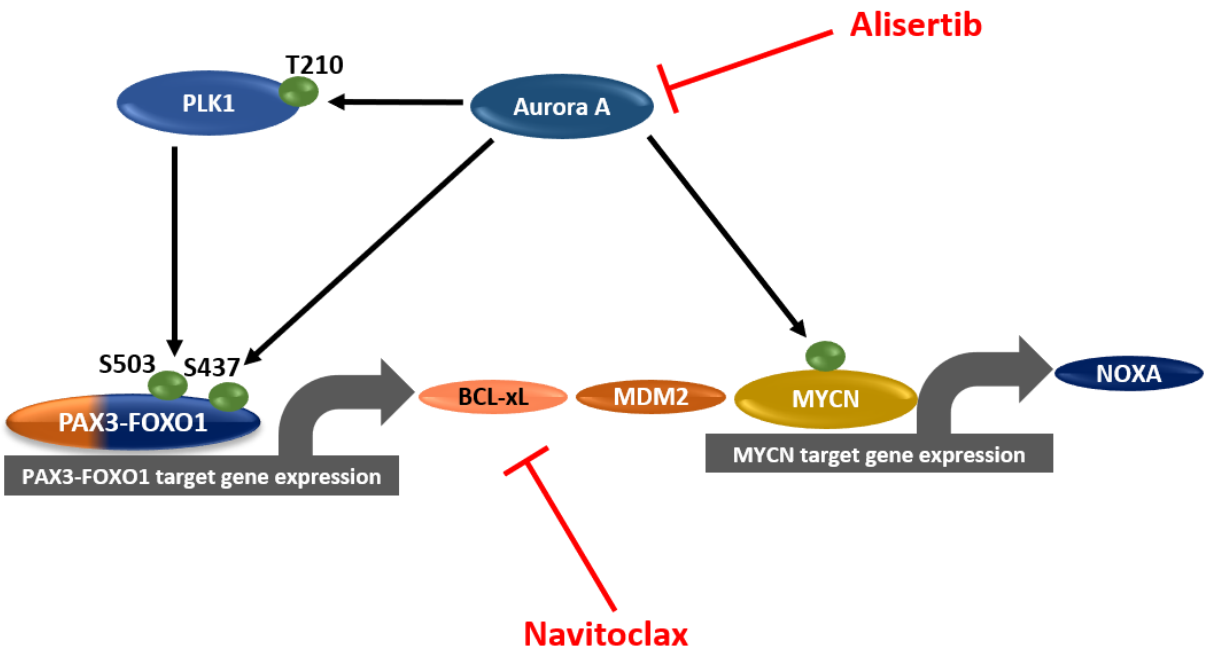
Supplemental Figure 7







Supplemental Figure 8



Supplemental Material and Methods

Site-directed mutagenesis

Site-directed mutagenesis was performed using the GeneArt Site-Directed Mutagenesis System (Thermo Fisher Scientific) according to manufacturer's protocol.

Immunoblot

Cells were lysed with RIPA buffer (see below), suspended in 4xLDS loading buffer (Thermo Fisher Scientific) supplemented with 250 mmol/L 1,4-Dithiothreitol (DTT, Sigma-Aldrich). After boiling the samples at 70°C for 5 minutes, proteins were separated by electrophoresis on 4-12% gradient polyacrylamide gels (Life Technologies). Proteins were transferred onto a Protran™ nitrocellulose membrane (GE Healthcare). Membranes were blocked in 5% (w/v) milk powder in Tris-buffered saline (TBS)/0.01% Tween20 (Sigma-Aldrich) and incubated overnight with primary antibodies at 4°C. Membranes were washed in TBS/Tween and incubated with horseradish peroxidase (HRP)-coupled IgG secondary antibody for 1h at room temperature. Membranes were washed in TBS/Tween and proteins were detected using ECL detection reagent (Thermo Fisher Scientific) in a ChemiDoc™ Touch Imaging system (BioRad).

RIPA buffer

Before use, buffer was supplemented with protease inhibitor (Roche Complete Mini)

Tris-Cl pH=7.5	50 mmol/L
Sodium chloride	150 mmol/L
NP-40	1%
Sodium deoxycholate	0.5%
Sodium dodecyl sulfate	0.1%
EGTA	1 mmol/L
Sodium fluoride	50 mmol/L
Beta glycerolphosphate	10 mmol/L
Sodium pyrophosphate	5 mmol/L
Sodium ortho vanadate	1 mmol/L

Co-Immunoprecipitation

Cells were lysed in ice cold Co-IP buffer (see below) and disrupted using syringes. Lysates were incubated with Protein G Dynabeads (Thermo Fisher Scientific)/antibody conjugates or beads alone for 3h at 4°C. Beads were washed and bound protein was released by boiling the beads in LDS buffer (as described above) at 70°C for 5 minutes.

CoIP buffer

Before use, buffer was supplemented with protease inhibitor (Roche Complete Mini)

Tris-Cl pH=7.5	50 mmol/L
Sodium chloride	80 mmol/L
NP-40	0.3%
Glycerol	10%
Magnesium chloride	1.5 mmol/L
Sodium fluoride	25 mmol/L
Beta glycerolphosphate	10 mmol/L
Sodium pyrophosphate	5 mmol/L
Sodium ortho vanadate	2 mmol/L

Generation of shRNA cell lines

Custom designed shRNAs in Lentiviral vectors (Cellecta) to transduce target cells with either the targeting shRNA plasmid (shP3F) or the non-targeting control shRNA plasmid (shscr) were ordered (Suppl.Fig.1A) (Cellecta):

HEK293T cells were co-transfected with the transfer plasmid, the envelope plasmid, and packaging plasmids. 48h after transfection viral supernatant was collected and concentrated using Amicon® Ultra Centrifugal Filters (Merck Millipore) according to manufacturer's protocol. Cell lines were transduced with viral concentrate in medium containing 8 μg/mL Hexadimethrine bromide (Sigma). Cells from PDXs were transduced without Hexadimethrine bromide. After 72h cells were selected with 0.5 μg/mL Puromycin (Sigma). In advance we tested three different shP3F sequences targeting the breakpoint region for knockdown efficiency and toxicity and continued with shP3F #3.

Targeting sequences

#1 CCTCTCACCTCAGAATTCAAT
#2 CTCTCACCTCAGAATTCAATT
#3 GGCCTCTCACCTCAGAATTCA

qRT-PCR

RNA was isolated from cells using the RNeasy Mini Kit (Qiagen). 1 µg of RNA was transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). Real-time PCR was performed using TaqMan probes (see below) and the TaqMan Gene Expression Master Mix (both Thermo Fisher Scientific). Expression changes were calculated using the ΔΔCt method with GAPDH as internal control.

TaqMan probes

All probes were ordered as Assay-on-Demand mix (Thermo Fisher Scientific) containing specific primers and FAM/BHQ-1 probe.

Gene	Assay ID
PAX3-FOXO1	Hs03024825
GAPDH	Hs02758991_g1
PMAIP1 (NOXA)	Hs00560402
MYCN	Hs00232074
MDM2	Hs01066930

Antibodies

Antigen	**Manufacturer**	**Host (Clone)**	**Dilution**
AURORA A	Genetex	ms mono (35C1)	1:1000
BAD			
BAK			
BAX			
BIM	Santa Cruz	ms mono (H-5)	1 :500
CASP9			
Cl. CASP3	Cell signaling	rb mono (D175)	1:1000
Cl. CASP7	Cell signaling	rb poly (D198)	1:1000
FOXO1	Santa Cruz	rb poly (H-128)	1:500
GAPDH	Cell signaling	rb mono (14C10)	1:1000
MYCN	Cell signaling	rb mono	1:1000
NOXA	Cell signaling	rb mono (D8L7U)	1:1000
P21	Cell signaling	rb mono (12D1)	1:1000
P53	Thermo Fisher Sc	ms mono (DO-1)	1:1000
PARP	Cell signaling	rb poly	1:1000
PLK1	Merck Millipore	ms mono	1:1000
pFOXO1$_{(S256 \triangleq S437)}$	Cell signaling		1:750
pPLK1$_{(T210)}$	Cell signaling		1:750
XIAP			

Supplemental Table 1: Drug library content

Product Name	Target
Veliparib (ABT-888)	PARP
Axitinib	VEGFR, PDGFR, c-Kit
Saracatinib (AZD0530)	Src, Bcr-Abl
FG-4592	HIF
Afatinib (BIBW2992)	EGFR
Bortezomib (PS-341)	Proteasome
Bosutinib (SKI-606)	Src
Dovitinib (TKI-258, CHIR-258)	c-Kit, FGFR, Flt, VEGFR, PDGFR
Dasatinib	Src, Bcr-Abl, c-Kit
Erlotinib HCl (OSI-744)	EGFR
Gefitinib (ZD1839)	EGFR
Lapatinib (GW-572016)	EGFR, HER2
Lenalidomide (CC-5013)	TNF-alpha
Nilotinib (AMN-107)	Bcr-Abl
Pazopanib HCl	VEGFR, PDGFR, c-Kit
Rapamycin (Sirolimus)	mTOR
Sorafenib Tosylate	VEGFR, PDGFR, Raf
Sunitinib Malate	VEGFR, PDGFR, c-Kit, Flt
Vandetanib (ZD6474)	VEGFR
Vorinostat (SAHA, MK0683)	HDAC
VX-680 (Tozasertib, MK-0457)	Aurora Kinase
Y-27632 2HCl	ROCK
Elesclomol (STA-4783)	HSP
Entinostat (MS-275)	HDAC
Enzastaurin (LY317615)	PKC
Olaparib (AZD2281, Ku-0059436)	PARP
GDC-0941	PI3K
SB431542	TGF-beta/Smad
Crizotinib (PF-02341066)	c-Met, ALK
AUY922 (NVP-AUY922)	HSP
PHA-665752	c-Met
SB216763	GSK-3
MK-2206 2HCl	Akt
Vismodegib (GDC-0449)	Hedgehog, P-gp
KU-55933 (ATM Kinase Inhibitor)	ATM
GSK1904529A	IGF-1R
MLN8054	Aurora Kinase
Danusertib (PHA-739358)	Aurora Kinase, FGFR, Bcr-Abl, c-RET, Src
GSK690693	Akt
JNJ-38877605	c-Met
Palbociclib (PD-0332991) HCl	CDK
Cabozantinib (BMS-907351)	VEGFR, c-Met, Flt, Tie-2, c-Kit
Everolimus (RAD001)	mTOR

BMS-754807	IGF-1R
YM155 (Sepantronium Bromide)	Survivin
Alisertib (MLN8237)	Aurora Kinase
AT9283	Bcr-Abl, JAK, Aurora Kinase
Barasertib (AZD1152-HQPA)	Aurora Kinase
Roscovitine (Seliciclib,CYC202)	CDK
Lenvatinib (E7080)	VEGFR
Valproic acid	GABA Receptor, HDAC
CYC116	Aurora Kinase, VEGFR
XAV-939	Wnt/beta-catenin
Thalidomide	Others
Decitabine	DNA/RNA Synthesis
PIK-75	PI3K, DNA-PK
2-Methoxyestradiol (2-MeOE2)	HIF
Vemurafenib (PLX4032, RG7204)	Raf
Rigosertib (ON-01910)	PLK
Ruxolitinib (INCB018424)	JAK
Resveratrol	Sirtuin
Ispinesib (SB-715992)	Kinesin
AEE788 (NVP-AEE788)	EGFR, Flt, VEGFR, HER2
PHA-793887	CDK
Ponatinib (AP24534)	Bcr-Abl, VEGFR, FGFR, PDGFR, Flt
AT7519	CDK
MK-1775	Wee1
Quizartinib (AC220)	Flt
AZD7762	Chk
R406 (free base)	Syk
Org 27569	Cannabinoid Receptor
EX 527 (Selisistat)	Sirtuin
Pomalidomide	TNF-alpha, COX
KU-60019	ATM
BIRB 796 (Doramapimod)	p38 MAPK
RO4929097	Y-Secretase
Tie2 kinase inhibitor	Tie-2
Azacitidine	DNA/RNA Synthesis
Acadesine	AMPK
Nicorandil	Others
PF-573228	FAK
Lovastatin	HMG-CoA Reductase
LDE225 (Erismodegib)	Smoothened
PF-4708671	S6 Kinase
MLN2238	Proteasome
MLN9708	Proteasome
SGI-1776 free base	Pim
AZ 960	JAK

Apatinib	VEGFR
Volasertib (BI 6727)	PLK
Degrasyn (WP1130)	DUB, Bcr-Abl
BKM120 (Buparlisib)	PI3K
Imatinib (STI571)	PDGFR,c-Kit, v-Abl
Mifepristone	Estrogen/progestogen Receptor
LY2603618	Chk
NU7441 (KU-57788)	DNA-PK, PI3K
MK-0752	Gamma-secretase
Trametinib (GSK1120212)	MEK
Ibrutinib (PCI-32765)	Src
NVP-BSK805 2HCl	JAK
GDC-0980 (RG7422)	mTOR, PI3K
A-769662	AMPK
AMG-900	Aurora Kinase
Crenolanib (CP-868596)	PDGFR
AZ 3146	Kinesin
PHA-767491	CDK
CUDC-907	HDAC, PI3K
NVP-BVU972	c-Met
SB705498	TRPV
Tofacitinib (CP-690550)	JAK
Dabrafenib (GSK2118436)	Raf
GDC-0068	Akt
Torin 2	mTOR
TAE226 (NVP-TAE226)	FAK
TPCA-1	IKK
Carfilzomib (PR-171)	Proteasome
T0070907	PPAR
WZ811	CXCR
IOX2	HIF
Evacetrapib (LY2484595)	CETP
Pazopanib	VEGFR
Rimonabant	Cannabinoid Receptor
Cabozantinib malate	c-met, VEGFR2
Spironolactone	Androgen Receptor
JNK-IN-8	Free Base
QNZ (EVP4593)	NF-κB
Tofacitinib (CP-690550) Citrate	JAK
GDC-0152	IAP
AZD3514	Androgen Receptor
AZ20	ATM/ATR
GSK126	Histone Methyltransferase
EPZ5676	Methyltransferase
GSK J4 HCl	Others

LDK378	ALK
IWP-2	Wnt/beta-catenin
GSK2334470	PDK-1
PF-3758309	PAK
HSP990 (NVP-HSP990)	HSP (e.g. HSP90)
AZD3463	ALK
EPZ-6438	Histone Methyltransferase
PYR-41	E1 Activating
PR-619	DUB
P5091 (P005091)	DUB
BMS-833923	Hedgehog/Smoothened
AZD1080	GSK-3
C646	Histone Acetyltransferase
10058-F4	c-Myc
AVL-292	BTK
IOX1	Histone demethylases
OG-L002	Histone demethylases
SGC-CBP30	Epigenetic Reader Domain
CNX-774	BTK
MM-102	Histone Methyltransferase
JIB-04	Histone demethylases
PFI-2	Histone Methyltransferase
CPI-203	Epigenetic Reader Domain
GSK2606414	PERK
6H05	Rho
K-Ras(G12C) inhibitor 9	Rho
SH-4-54	STAT
OTX015	BET
LEE011	CDK
LDC000067	CDK
PI-1840	Proteasome
JNK Inhibitor IX	JNK
GNF-5837	Trk receptor
Afuresertib (GSK2110183)	Akt
GDC-0994	ERK
UNC0379	Histone Methyltransferase
GSK-LSD1 2HCl	Histone Demethylase
GSK J1	Histone Demethylase
INCB024360	IDO
BRD4770	Histone Methyltransferase
BV-6	IAP
EI1	Histone Methyltransferase
MI-2 (Menin-MLL Inhibitor)	Histone Methyltransferase
LDC1267	Axl
CPI-360	Histone Methyltransferase
CH5183284 (Debio-1347)	FGFR
YK-4-279	DNA/RNA Synthesis

AZD6738	ATM/ATR
Verdinexor (KPT-335)	CRM1
EPZ015666	Histone Methyltransferase
Pexmetinib (ARRY-614)	p38 MAPK
Pexidartinib (PLX3397)	CSF-1R
BI-847325	MEK
PFI-4	Epigenetic Reader Domain
Epacadostat (INCB024360)	IDO
NSC 23766	Rac
BMS-345541	IκB/IKK
Pacritinib (SB1518)	JAK
Idasanutlin	MDM2/p53
iBet762	BET
ABT-263	BCL2, BCL-XL, BCL-w
ABT-199	BCL2
Obatoclax	BCL2-family
Dynasore	Dynamin
Dyngo4a	Dynamin
GDC-0973	MEK
Fenretinide	Retinoid Acid Receptor
JQ-1	BET
Birinapant	IAP
Doxorubicine	DNA
Vincristine	Microtubuli
Etoposide	Topoisomerase

6.2 MANUSCRIPT II

MDM2 is a putative novel target gene of PAX3-FOXO1 in alveolar rhabdomyosarcoma and contributes to cell survival in patient-derived xenografts

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Conflict of interest: The authors declare no conflict of interest.

Manuscript in preparation.

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Author contributions: JO, MD, and JGM acquired data. JO, JGM, and MW contributed to the analysis and interpretation of data. JO, MD, MW, and BWS made substantial contributions to the conception, design, and intellectual content of the paper. JO and BWS wrote the paper

Abstract

Alveolar rhabdomyosarcoma (aRMS) is an aggressive subtype of the most common soft tissue sarcoma in children. 5-year overall survival in aRMS patients is as low as 30%. High tendency to metastasize and resistance to standard of care therapy are factors contributing to the malignancy of this cancer. The major driver of tumorigenesis and maintenance in aRMS is the fusion protein PAX3-FOXO1, a strong transcriptional activator. Knowledge of how PAX3-FOXO1 contributes to the ability of aRMS cells to evade cell death could provide novel insights. Recently, we demonstrated that PAX3-FOXO1 depletion results in NOXA-dependent apoptosis. Our aim here was therefore, to investigate the PAX3-FOXO1-dependent regulatory network of NOXA to find new tumor-specific vulnerabilities.

Here, using gene expression analysis and chromatin immunoprecipitation sequencing (ChIP-Seq) experiments we show that in aRMS cell lines NOXA expression is activated by MYCN, a PAX3-FOXO1 target gene. Furthermore, using ChIP-Seq analysis we found a novel putative regulation of the p53 regulator MDM2 by the fusion protein. Accordingly, patient-derived xenograft cells from aRMS patients are sensitive to pharmacological disruption of the MDM2/p53 interaction using Idasanutlin.

Taken together, these data shed light on the regulatory pathways controlling NOXA expression in aRMS cell and offer novel opportunities for cell death-directed therapy.

Keywords: PAX3-FOXO1, MYCN, NOXA, MDM2, Idasanutlin

Introduction

Rhabdomyosarcoma (RMS) is the most common soft tissue sarcoma in children. The alveolar subtype is the most aggressive form of RMS, showing a high tendency to metastasize. Patients with metastatic aRMS have a 5-year overall survival rate of 30% [1]. Molecularly, aRMS tumors are characterized by reciprocal translocation resulting in the expression of oncogenic PAX-fusion proteins [2]. The most common fusion protein is PAX3-FOXO1 and has been shown to be essential for tumor maintenance [3,4]. Therapeutic options for aRMS patients comprise surgery, radiation, and chemotherapy, which cannot overcome the dismal prognosis [5].

Resistance towards standard-of-care therapy is one of the major problems in clinics today and calls for novel approaches targeting cancer-specific vulnerabilities [6]. Evasion from cell death is one of these cancer-specific traits, a hallmark of cancer [7]. Thus, knowledge of the molecular basis of cell death resistance could help improve outcome of both standard-of-care therapies and potential novel targeted approaches. Typically, mutations and oncogene overexpression result in activation of cell-intrinsic countermeasures preventing proliferation or inducing programmed cell death [8]. Thus, cancer cells have to develop strategies to circumvent these intrinsic anti-cancer mechanisms.

The tumor suppressor p53 integrates stress signals such as DNA-damage, metabolic stress, translational stress and activates adequate responses, such as cell cycle arrest or apoptosis. Here, pro-apoptotic proteins are transcriptionally upregulated and localize at the outer mitochondrial membrane (OMM) [8]. At the OMM, pro-apoptotic BH3-only proteins, such as NOXA exist in an equilibrium state with anti-apoptotic proteins of the BCL-2 family [9]. A shift of this equilibrium towards pro-apoptotic results in activation of BAX and BAK which homodimerize and form a pore in the OMM thus initiating the apoptotic caspase-cascade [8].

In many cancers one way to prevent apoptosis therefore is to upregulate anti-apoptotic BCL-2 proteins, increasing the threshold of apoptosis induction [9]. Another way for cancer cells to avoid apoptosis is to interfere with p53 activity [10]. P53 stability regulated by the ubiquitin-ligase MDM2. The MDM2 locus was found to be amplified in certain RMS tumors which results in reduced p53 functionality [11]. In aRMS tumors PAX3-FOXO1 was shown to be critical for tumor cell survival [4]. Recently, our group could demonstrate that knockdown of PAX3-FOXO1 leads to intrinsic apoptosis in aRMS cells, dependent on the BH3-only protein NOXA (unpublished). However, exactly how PAX3-FOXO1 regulates NOXA, could not be established.

Therefore, in this follow-up study we aimed to characterize the mechanisms by which PAX3-FOXO1 regulates NOXA and thereby prevents cell death.

Here, we show that NOXA expression is upregulated upon knockdown of PAX3-FOXO1 and that MYCN is the major driver of NOXA expression under basal conditions. Furthermore, we found indications for a direct transcriptional regulation of MDM2 by PAX3-FOXO1. Consequently, we demonstrate that aRMS cancer cells from patient-derived xenografts were sensitive to pharmacological disruption of MDM2/p53 interaction.

Results

NOXA expression is upregulated after silencing of PAX3-FOXO1

Our recent studies revealed that after silencing of PAX3-FOXO1 cells undergo intrinsic apoptosis dependent on NOXA. We engineered aRMS cell lines to express either small hairpin RNA targeting PAX3-FOXO1 mRNA (shP3F) or a non-targeting control (shsc). To gain further insights into the regulation of pro-apoptotic mechanisms in aRMS, we analyzed differential expression of NOXA in RNA-Seq data of both Rh4 and Rh30. In both Rh4 and Rh30 cells we observed upregulation of NOXA by 1.3-fold, 24h after knockdown of PAX3-FOXO1 and by 1.8-fold after 48h in Rh4 (Fig. 1A, Suppl. Fig. 1A-B). We confirmed this upregulation in Rh4 cells using qRT-PCR and western blots. 48h after shRNA-mediated silencing of PAX3-FOXO1 NOXA mRNA expression increased 2.8-fold compared to the scrambled control (shsc) (Fig. 1B). Knockdown of PAX3-FOXO1 mRNA resulted in loss of PAX3-FOXO1 protein levels and concomitant increased wild-type FOXO1 (Fig. 1C). We also confirmed increased NOXA protein levels (Fig. 1C). To confirm these findings in other cell lines, we analyzed NOXA protein expression by western blot. Here, we found that in three out of four aRMS cell lines 48h after PAX3-FOXO1 knockdown, NOXA protein levels were 2-4-fold higher (Fig. 1D). Only Rh30 cells showed no upregulation of NOXA. Next, we wanted to confirm these findings in primary tumor material. To this end, we cultivated cells obtained from patient-derived xenografts (PDX) and stably transduced them to express the inducible shRNA system described above. As shsc showed toxicity in MAST118 PDX cells (data not shown), we utilized shRNA directed against luciferase (shluc) as a control. We confirmed knockdown of PAX3-FOXO1 by western blot 48h after shRNA induction and observed increased levels of NOXA protein, as well as cleaved caspase 7 (Fig. 1E), indicating that these cells react the same ways as the cell lines used above: by initiating apoptosis after silencing of PAX3-FOXO1.

Taken together, these findings show that NOXA expression is upregulated after silencing of PAX3-FOXO1 not only in cell lines but also in PDX-derived cells.

MYCN drives NOXA expression in aRMS cells

Next, we aimed to characterize NOXA regulation in aRMS. To this end, we first established whether PAX3-FOXO1 could bind directly to the PMAIP1. ChIP-Seq data from Rh4 cells revealed that there was no enrichment of PAX3-FOXO1 binding to the PMAIP1 promoter, neither in Rh4 cells lines nor in MAST118 cells from patient-derived xenografts (Fig 2A). These findings indicate that NOXA expression is not directly regulated by PAX3-FOXO1.

Therefore, we aimed to find potential direct regulators of NOXA expression that could contribute to increased NOXA expression after silencing of PAX3-FOXO1. To this end, we used both the Rh4 and RHjT cell lines expressing the inducible shRNA constructs. In these cells we simultaneously induced shRNA and used siRNA mediated knockdown of factors known to regulate NOXA expression in other cells: We focused our attention on p53 and wild-type FOXO1. We chose p53, as it is the most characterized regulator of NOXA expression and FOXO1 because we observed upregulation of FOXO1 after silencing of PAX3-FOXO1 indicating that it might play a role in contributing to cell death. Western blot analysis showed that neither knock down of p53 nor FOXO1 respectively, abrogated NOXA expression (Fig. 2B-C, Suppl. Fig. 2A). We also investigated the effects of siRNA mediated knock downs on caspase 3/7 activity to establish the influence on cell death. Neither p53 knock down nor FOXO1 knockdown rescued aRMS cells from apoptosis after silencing of PAX3-FOXO1 (Fig. 2D-E, Suppl. Fig. 2B-C). These findings indicate that in cell lines neither p53 nor FOXO1 contribute substantially to NOXA upregulation after silencing of PAX3-FOXO1. A recent study demonstrated that NOXA expression is driven by MYCN in neuroblastoma [12]. Therefore, we investigated whether such regulation could also be observed in aRMS cells. Indeed, analyzing ChIP-Seq data from Rh4 cells, we observed enrichment of MYCN at the PMAIP1 promoter in addition to peaks of both H3K4me3 and H3K27ac, indicating active transcription and not repression by MYCN (Fig 2A). Furthermore, siRNA-mediated knockdown of MYCN abrogated NOXA protein levels in both aRMS cell lines (Fig. 2B-C). Additional silencing of PAX3-FOXO1 lead to an increase of NOXA protein expression, albeit far weaker than after knockdown of p53 or FOXO1 (Fig. 2B-C, Suppl. Fig. 2A), indicating that MYCN is the major driver of NOXA expression in aRMS. Depletion of MYCN rescued RHJT cells from cell death (Fig. 2E), however it did not rescue Rh4 cells from cell death (Fig. 2D).

These findings demonstrate that MYCN binds to the PMAIP1 promoter in aRMS cell lines and positively regulates NOXA expression. However, the exact contribution of MYCN to cell death after silencing of PAX3-FOXO1 remains to be demonstrated.

MDM2 is a potential new target gene of PAX3-FOXO1 but has no influence on NOXA expression

During our investigations we analyzed RNA-Seq data and found that expression of MDM2, a negative regulator of p53 stability, decreased after silencing of PAX3-FOXO1 in Rh4 cells (Fig. 1A, Suppl. Fig 1A-B). In Rh30 cells after 24h, this decrease was comparable to that of MYCN, a known target gene of PAX3-FOXO1 (Suppl. Fig 1B). In Rh4 cells, both after 24h and 48h the decrease of MDM2 expression was even more pronounced as that of MYCN (Fig. 1A, Suppl. Fig 1A). These findings led us to hypothesize that MDM2 expression might be regulated by PAX3-FOXO1. We analyzed MDM2 mRNA expression by qRT-PCR after silencing of PAX3-FOXO1 in 5 different aRMS cell lines and found that MDM2 expression was reduced in all samples after 48h. The Rh4 and KFR cell lines showed significant reductions to under 40% compared to shsc (Fig. 3A). We observed the same effects on protein levels of MDM2 after silencing of PAX3-FOXO1 (Fig. 3B). To investigate whether MDM2 expression was directly regulated by the fusion protein we analyzed ChIP-Seq data and found that in Rh4 cells but not in MAST118 cells PAX3-FOXO1 binding is enriched at the P1 promoter region of the MDM2 gene in concert with marks for active transcription (Pol II binding, histone marks) (Fig. 3C). Interestingly, we also found enrichment of MYCN at the P1 promoter of MDM2 (Fig. 3C). This finding suggests that PAX3-FOXO1 drives MDM2 expression. We hypothesized that MDM2 as a potential target gene of PAX3-FOXO1 could destabilize p53 and thus negatively affect NOXA expression. Conversely, siRNA-mediated knockdown of MDM2 (Suppl. Fig. 3A-B) had only minor to no effects on NOXA expression in Rh4 cell or RHjT cells, respectively (Fig. 3D). These data indicate that in aRMS cell lines MDM2 does not affect NOXA expression.

Lastly, like most cell lines, Rh4 and RHjT cell lines are deficient for p53 signaling [13]. Therefore, we reasoned that these cell lines might not reflect the biology of primary aRMS tumors which are almost exclusively known to express wild-type p53 [11]. To investigate the implications of MDM2 as a putative new target gene of PAX3-FOXO1, we analyzed whether aRMS cells derived from PDXs were susceptible to pharmacological disruption of the MDM2/p53 interaction. Indeed, treatment with increasing concentrations of Idasanutlin in MAST60 and MAST118 cells resulted in stabilization of p53 and increased protein levels of p21 and cleaved caspase 7, indicating active apoptosis (Fig. 3E-F). As

expected, Rh4 cells were not sensitive at all to Idasanutlin treatment (Fig.3G). To exclude that this treatment could affect non-tumor cells, we treated immortalized myoblasts. Here, Idasanutlin treatment also resulting in p53 stabilization, however caspase activation was not detected (Fig. 3H).

Discussion

In this study, we aimed to characterize mechanisms driving NOXA expression in aRMS cells. Here, we show that, conversely not classical tumor suppressor proteins, such as p53 or FOXO1, drive NOXA expression but MYCN, an oncogene. Furthermore, we identified MDM2 as a potential new target gene of PAX3-FOXO1 which makes primary aRMS tumor cells sensitive to pharmacological disruption of MDM2/p53 interaction.

We found that NOXA was upregulated after silencing of PAX3-FOXO1 indicating that the fusion protein while active suppresses NOXA expression. While we could confirm this connection in 5 cell lines, we were only able to generate transgenic clones of one patient-derived xenograft, MAST118. Given the large heterogeneity among rhabdomyosarcoma tumors, these results would need to be bolstered by experiments with further PDX samples. A major complication for this idea however, is that primary patient materials are hard to come by, given the few aRMS cases occurring per year [14]. Furthermore, it is complicated to establish the perfect conditions for PDX-derived cells to grow in culture without losing its primary tumor traits [15,16].

Interestingly, we found that MYCN drives NOXA expression in aRMS cell lines. This phenomenon was recently described for neuroblastoma, where MYCN amplification is common [12]. While, we did observe the high dependency of NOXA expression on MYCN both in the Rh4 and RHjT cell line, this regulation seems to contribute to apoptosis only in RHjT cells. In Rh4 cells knockdown of MYCN could not rescue the cells from cell death after silencing of PAX3-FOXO1, indicating that both cell lines differ in their apoptotic potential. One major difference is, that RHjT cells are known to harbor MYCN amplification. Thus, this cell line might rely more on MYCN function and already express higher levels of NOXA, making them primed for apoptosis. To compensate for higher NOXA levels, RHjT cells would need to express higher levels of anti-apoptotic BCL-2 proteins to increase the apoptotic threshold. Loss of MYCN expression and resulting lower levels of NOXA could therefore result in anti-apoptotic proteins outnumbering the pro-apoptotic factors leading to less apoptosis. Here it would be interesting to analyze the expression levels of anti-apoptotic proteins, like MCL-1, the canonical interactor of NOXA [17], between different cell lines to explain differences in apoptotic priming. Furthermore, the subgroup of

MYCN-amplified aRMS tumors might therefore be sensitive to therapy with BH3-mimetics, like ABT-199 or ABT-263 which would provide clinicians with targeted therapy options to combine with standard-of-care therapy.

Although our findings provide a mechanism of NOXA regulation in aRMS under basal conditions, the results are contradictory when it comes to NOXA-dependent cell death after silencing of PAX3-FOXO1. Silencing of PAX3-FOXO1 results in reduced expression of its target genes, which also contain MYCN. Lower levels of MYCN result in lower levels NOXA, as we could demonstrate using siRNA. However, what we observe is, that silencing of PAX3-FOXO1 results in strong upregulation of NOXA despite abrogated MYCN expression. Here clearly, we have not characterized all mechanisms driving NOXA expression in aRMS. There must be another regulator that is potentially shut down by PAX3-FOXO1 and therefore only becomes active after silencing of the fusion protein. Finding this elusive regulator would require unbiased screening approaches with cells being deficient for individual candidates. However, NOXA is a protein that is commonly upregulated upon a wide variety of cellular stresses [18]. Therefore, many factors can contribute to its expression and increased NOXA expression after silencing of PAX3-FOXO1 might simply reflect cellular stress after loss its critical oncogene.

Lastly, we identified MDM2 as a putative new target gene of PAX3-FOXO1. This relation has not been demonstrated so far and provides insight into further options how aRMS could avoid apoptosis. We demonstrate a fusion protein dependent expression of MDM2 which alone would not allow the conclusion of a direct regulation. ChIP-Seq analysis provides evidence of PAX3-FOXO1 binding to the promoter region of the MDM2 locus and an active signature. However, these results were only obtained from one cell line and the results from the PDX tumor differ. Our findings thus might not reflect the majority aRMS tumors. Therefore, it would be necessary to prove this direct regulation using further cell lines or better PDX cells. An indication for the validity of our findings is, that the P1 promoter region of MDM2 contains a PAX3-FOXO1 consensus motif which also overlaps with the ChIP-Seq binding peak (Suppl. Fig. 3D). Interestingly, we also found binding of MYCN at the P1 promoter of MDM2 indicating that also MYCN drives MDM2 expression. This MYCN-dependent regulation of MDM2 has already been described in neuroblastoma [19,20]. In the context of aRMS this finding indicates that apart from a potential direct regulation of MDM2 by PAX3-FOXO1, this regulation could also be indirect via MYCN as a target gene of PAX3-FOXO1. Thus, to conclusively support the hypothesis of direct MDM2 regulation by the fusion protein, more functional tests, for instance luciferase promoter assays, need to be established.

Although MDM2 was not connected to NOXA expression in the cell lines we investigated, in primary or PDX derived cells the MDM2/p53 is still intact and might well contribute to NOXA expression. Thus, upregulation of MDM2 by PAX3-FOXO1 could indicate at an important role of MDM2 in preventing cell death in aRMS tumors. In other rhabdomyosarcoma tumors the MDM2 locus is often found to be amplified [11] further underpinning the important role of MDM2 as an oncogene in rhabdomyosarcoma. We therefore reasoned that primary aRMS tumors might respond to pharmacological disruption of the MDM2/p53 interaction. Indeed, in line with our hypothesis, cells from MAST60 and MAST118 PDX tumors were sensitive to Idasanutlin treatment in vitro. We demonstrate, for the first time the sensitivity of patient-derived aRMS cells to this treatment.

Taken together, we demonstrate that NOXA expression is driven by MYCN in aRMS cell lines. Furthermore, we show PAX3-FOXO1 dependent regulation of MDM2 expression and sensitivity of aRMS PDX tumors to MDM2-directed therapy strategies which could provide further combination options to boost efficiency of standard-of-care treatment.

Material and Methods

Cell culture

Cell lines: Rhabdomyosarcoma cell lines RD, Rh4 (both received from Peter Houghton, St. Jude Children's Hospital, Memphis, TN), RhJT (Scott Diede, Fred Hutchinson Cancer Research Center, Seattle, WA), RMS (Janet Shipley, Sarcoma Molecular Pathology, The Institute of Cancer Research, London, UK), KFR (Jindrich Cinatl, Frankfurter Stiftung für krebskranke Kinder, Frankfurt, Germany), and Rh30 (ATCC LGC Promochem) were cultured in high glucose DMEM (Sigma-Aldrich), supplemented with 100 U/mL penicillin/streptomycin, 2 mmol/L L-glutamine, and 10% FBS (Life Technologies) at 37°C and 5% CO_2 . Immortalized myoblasts (Vincent Mouly, UPMC Université de Paris, France) [21] were cultured in Skeletal Muscle Cell Basal Medium (Life technologies) supplemented with 10 ng/ml EGF and 0.1 ng/ml bFGF (both Peprotech Inc.).

PDX cells: PDX tumors were obtained from St.Jude's Children's Hospital, Memphis dissociated as described before [22]. In brief, tumor tissue was minced with scalpels under sterile conditions and suspended in Hanks' Balanced Salt Solution (HBSS, Sigma-Aldrich) supplemented with 1 mmol/L $MgCl_2$, 200 µg/mL Liberase, and 200 U/mL DNase I (both Roche). Tissue pieces were digested for 30 minutes at 37°C and filtered twice through 70 µm cell strainers (BD Biosciences). Dissociated cells were washed with phosphate-buffered saline (PBS, Sigma-Aldrich) before freezing or resuspending for further culture.

Both MAST60 and MAST118 cells derived from the respective PDX tumors were cultured in Neurobasal medium (Life Technologies) supplemented with 2x B-27™ Supplement (Life Technologies), 20 ng/mL EGF and 20 ng/mL basic FGF (Peprotech) on plates coated with Matrigel® (Corning Life Sciences).

Virus production and transduction

Lentiviral particles (for stable integration of shRNA) were produced in HEK293T cells. In brief, cells were transfected with Pax2 and VSV-G plasmids (both Addgene #12259) as well as with the respective transfer plasmid using calcium phosphate transfection. Virus containing supernatant was concentrated using Amicon Ultra centrifugal filter units (Merck Millipore). aRMS cell lines were transduced with the virus particles in presence of 8 µg/mL hexadimethrine bromide (Polybrene, Sigma-Aldrich). Transduced cells were selected with 0.5 µg/mL puromycin). MAST118 cells were transduced without Polybrene.

RNA-Sequencing

Inducible shRNA was induced in Rh4 cells with 0.1 µg/mL doxycycline. After 48h and 72h RNA was extracted and processed for RNA-Seq analysis as described before [23].

Chromatin immunoprecipitation (ChIP)-Seq

Chromatin immunoprecipitation and subsequent sequencing analysis was performed as described before [23]. In brief: ChIP assays were performed by using the ChIP-IT High Sensitivity kit (#53040, Active Motif) according to the manufacturer's instructions. Briefly, cells were grown to confluence, fixed with 1% formaldehyde (Sigma-Aldrich) for 13 min, harvested and sonicated with the EpiShear™ ProbeSonicator (#53052, Active Motif) for 27 cycles (30% amp, 30sec ON, 30sec OFF). Sonicated lysates were then quantified and 30ug of chromatin were incubated overnight at 4°C with 5-10ug of antibody (antibodies listed in Supplementary Table 5). DNA was purified according to the manufacturer's instructions.

TaqMan qRT-PCR

RNA was isolated from cells using the RNeasy Mini Kit (Qiagen). 1 µg of RNA was transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). Real-time PCR was performed using TaqMan probes (Supplemental Materials) and the TaqMan Gene Expression Master Mix (both Thermo Fisher Scientific). Expression changes were calculated using the ΔΔCt method with GAPDH as internal control.

Immunoblot

Cells were lysed with RIPA buffer, suspended in 4xLDS loading buffer (Thermo Fisher Scientific) supplemented with 250 mmol/L 1,4-Dithiothreitol (DTT, Sigma-Aldrich). After boiling the samples at 70°C for 5 minutes, proteins were separated by electrophoresis on 4-12% gradient polyacrylamide gels (Life Technologies). Proteins were transferred onto a Protran™ nitrocellulose membrane (GE Healthcare). Membranes were blocked in 5% (w/v) milk powder in Tris-buffered saline (TBS)/0.01% Tween20 (Sigma-Aldrich) and incubated overnight with primary antibodies at 4°C. Membranes were washed in TBS/Tween and incubated with horseradish peroxidase (HRP)-coupled IgG secondary antibody for 1h at room temperature. Membranes were washed in TBS/Tween and proteins were

detected using ECL detection reagent (Thermo Fisher Scientific) in a ChemiDoc™ Touch Imaging system (BioRad).

Antibodies are described in the Supplemental Materials and Methods section.

siRNA transfection

Cells were reverse transfected at the time of seeding: 1.13 µL siRNA (stock: 5 µmol/L) (Thermo Fisher Scientific, Supplemental Materials) were thoroughly mixed with 11.3 µL of Interferin® (Polyplus) in 150 µL serum-free medium, incubated for 15 minutes at room temperature and added to the wells. Cells were detached using 0.05% Trypsin/EDTA (Thermo Fisher Scientific), adjusted to the appropriate number and seeded onto the wells.

Caspase activity assay

Caspase activity was assessed using Caspase-Glo® 3/7 Assay (Promega) according to manufacturer's protocol. In brief, Assay solution was added directly to each well, plate was covered and shaken, followed by incubation at room temperature for 45 minutes. Signal was assessed by measuring chemiluminescence.

Statistics

Statistical analysis was performed using GraphPad Prism 7. Welch's two-tailed test and Mann-Whitney test were used for comparisons. Statistical significance was given at $p<0.05$.

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Figure legends

Figure 1: NOXA is upregulated after silencing of PAX3-FOXO1

A. Comparative RNA-Seq expression data. FPKM values were analyzed and compared from both Rh4shsc (x-axis) and Rh4shP3F (y-axis) cells 48h after induction of shRNA with 0.1 µg/mL doxycycline. PMAIP1 (NOXA) indicated as green dot, MDM2 indicated as red dot. Lower panel: Change of FPKM values for MDM2 (red), PMAIP1 (NOXA, green), and MYCN (blue) in Rh4shsc and Rh4shP3F cells after 48h **B.** Relative mRNA expression of NOXA in Rh4 shsc or shP3F 48h after induction of shRNA with 0.1 µg/mL doxycycline. Gene expression was normalized to GAPDH. Mean of two independent experiments performed in triplicates; bars, SD; Welch's two-tailed t-test; **, $P \leq 0.01$ **C.** Immunoblot for NOXA protein levels in both Rh4 shsc and Rh4 shP3F cells 48h after induction of shRNA. Representative blot of at least three independent experiments **D.** NOXA protein expression in the given cell lines with scrambled shRNA (shsc) or shRNA targeting PAX3-FOXO1 (shP3F), 48h after induction of shRNA (+) or non-induced control (-). Upper panel: Immunoblots for NOXA and GAPDH, lower panels: relative densitometric quantification of protein bands, relative to GAPDH. **E.** Immunoblots for lysates of MAST118 cells stably expressing either shLuciferase (shluc) or shP3F inducible constructs. 48h after induction or no induction.

Figure 2: NOXA expression is not regulated by FOXO1 but by MYCN

A. ChIP-Seq analysis of protein binding at the PMAIP1 locus. DNA from both Rh4 and MAST118 cells was isolated and incubated with the respective antibodies to immunoprecipitate bound DNA motifs. Sequencing analysis for enriched binding of the histone mark or protein at the PMAIP1 locus. PAX3-FOXO1: red lane, all other lanes: from Rh4: MYCN: dark blue lane, Pol II: Polymerase II **B-C.** Immunoblots for lysates from Rh4shP3F (B) or RHJTshP3F (C) cells 48h after induction of shP3F (+) or no induction (-). Additionally, cells were transfected with siRNA targeting FOXO1, MYCN (N-MYC) or not targeting (scr). **D-E.** Relative caspase 3/7 activity measured by Caspase 3/7 Glo® in Rh4sh cells (D) or RHJTsh cells (E) 48h after induction of shRNA (+) or no induction (-). Additionally, cells were transfected with siRNA against FOXO1 or MYCN, or with either non-targeting siRNA (scr) or no transfection at all (Ctrl). Mean of four independent experiments performed in triplicates; bars, SD; Mann-Whitney-test, *, $p < 0.05$

Figure 3: MDM2 is a potential new target gene of PAX3-FOXO1

A. MDM2 mRNA expression is reduced after silencing of PAX3-FOXO1. mRNA expression was analyzed by qRT-PCR in transgenic 5 cell lines either expressing scrambled shRNA (dark red) or shP3F (light red) 48h after induction with 0.1 µg/mL doxycycline. Values indicate the fold-change compared to uninduced control. Mean of three independent experiments performed in triplicates; bars, SD; Mann-Whitney-test; *, $p < 0.05$; **, $p < 0.01$ **B.** Immunoblots for the same cell lines 48h after shRNA induction. **C.** ChIP-Seq analysis of the gene locus. Upper panel: chromosome map indicating the location of the MDM2 gene. Lower panel: enrichment of histone marks or protein binding indicated by peaks, red: PAX3-FOXO1 (from cells as indicated), all other lanes from Rh4: dark blue: MYCN, Pol II: Polymerase II. **D.** Immunoblot for MDM2, NOXA and GAPDH of lysates from both Rh4shP3F and RHJTshP3F cells with or without induction of shRNA as indicated. Additionally, cells were transfected with siRNA against MDM2 or non-targeting siRNA (scr) **E-H.** Immunoblots of lysates from PDX-derived cells (E+F), Rh4 cell line (G), or immortalized myoblasts (H). Cells were treated for 48h with increasing concentrations of Idasanutlin.

Supplemental Figure 1

A-B. RNA-Seq data from aRMS cell lines expressing either scrambled shRNA (shsc) or shRNA against PAX3-FOXO1 (shP3F) 24h after shRNA induction with 0.1 µg/mL doxycycline. A. Rh4 cells B. Rh30 cells. Right panels: individual change of FPKM values for the respective genes after 24h in Rh4 cells (A) or Rh30 cells (B).

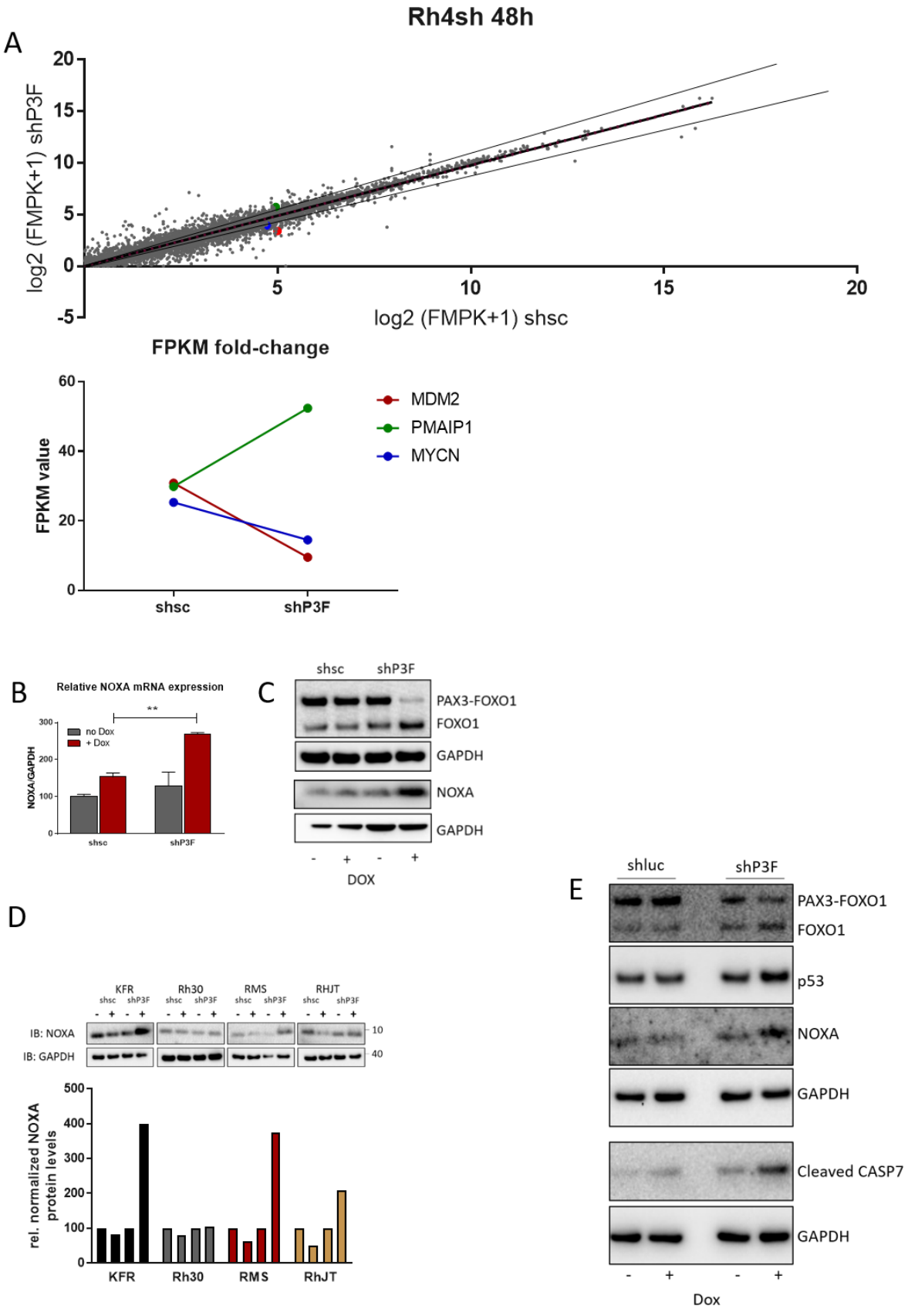
Supplemental Figure 2

A. Immunoblot of Rh4shP3F (left panel) and RHJTshP3F cells (right panel) 48h after shRNA induction (+) or no induction (-). Additionally, cells were transfected with either non-targeting siRNA (siscr) or siRNA targeting p53. B-C. Caspase activity in Rh4shP3F cells (B) or RHJTshP3F cells (C) 48h after induction of shRNA (black bars) or no induction (white bars). Additionally, cells were transfected with the given siRNAs or siRNA combinations. Mean of two experiments performed in triplicates; bars, SD.

Supplemental Figure 3

A. Immunoblot to confirm siRNA-mediated knockdown of MDM2 in Rh4shP3F or RHJTshP3F cells, respectively. Ctrl: untreated cells (no siRNA). 48h after induction of shRNA with doxycycline (+) or no induction (-). B. Binding motif of PAX3-FOXO1

Figure 1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Rh4sh 48h
log2 (FMPK+1) shP3F
log2 (FMPK+1) shsc
FPKM fold-change
FPKM value
MDM2
PMAIP1
MYCN
shsc
shP3F
B
Relative NOXA mRNA expression
NOXA/GAPDH
no Dox
+ Dox
C
shsc
shP3F
PAX3-FOXO1
FOXO1
GAPDH
NOXA
GAPDH
DOX
D
KFR
Rh30
RMS
RHJT
IB: NOXA
IB: GAPDH
rel. normalized NOXA protein levels
E
shluc
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PAX3-FOXO1
FOXO1
p53
NOXA
GAPDH
Cleaved CASP7
GAPDH
Dox

Figure 2

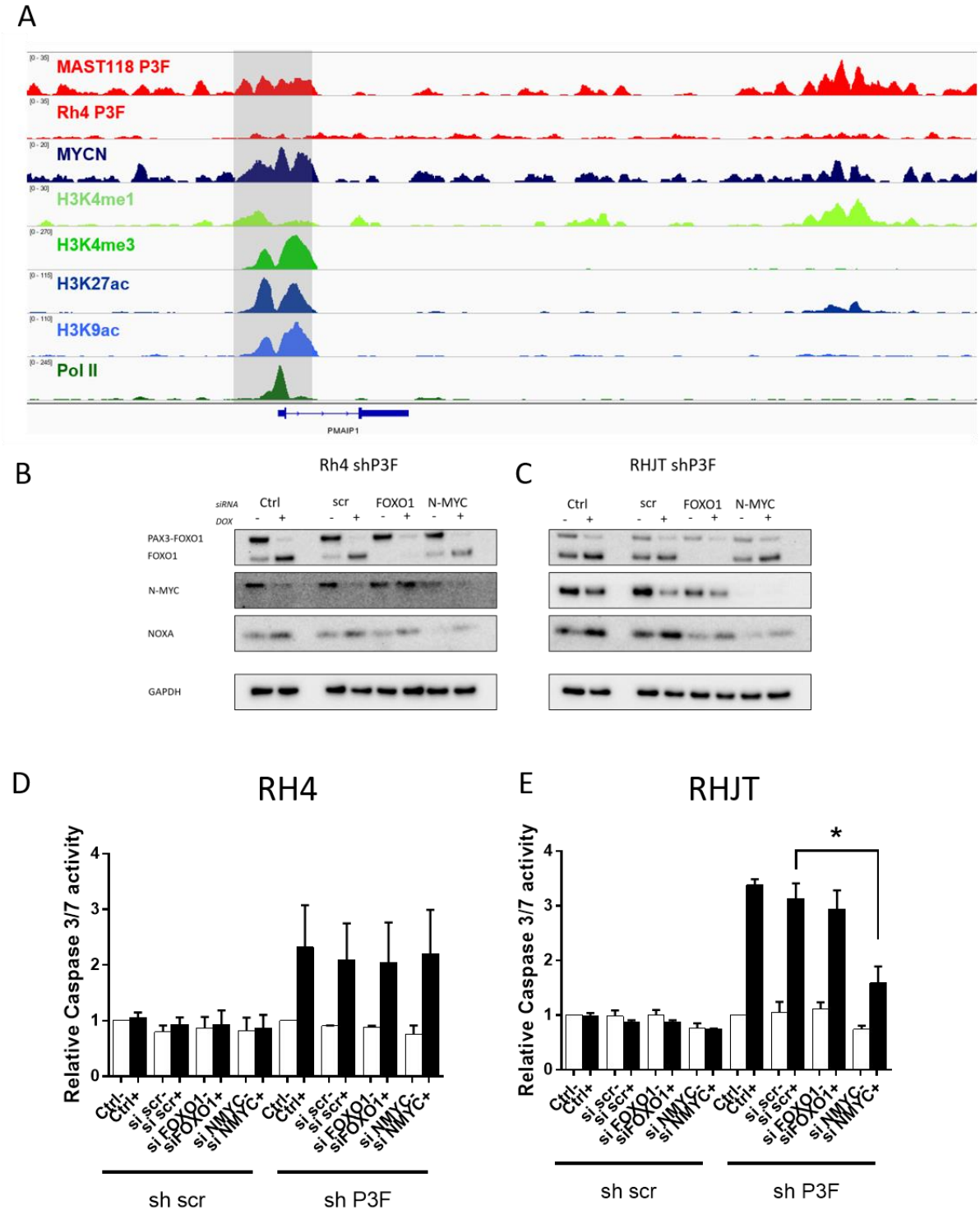
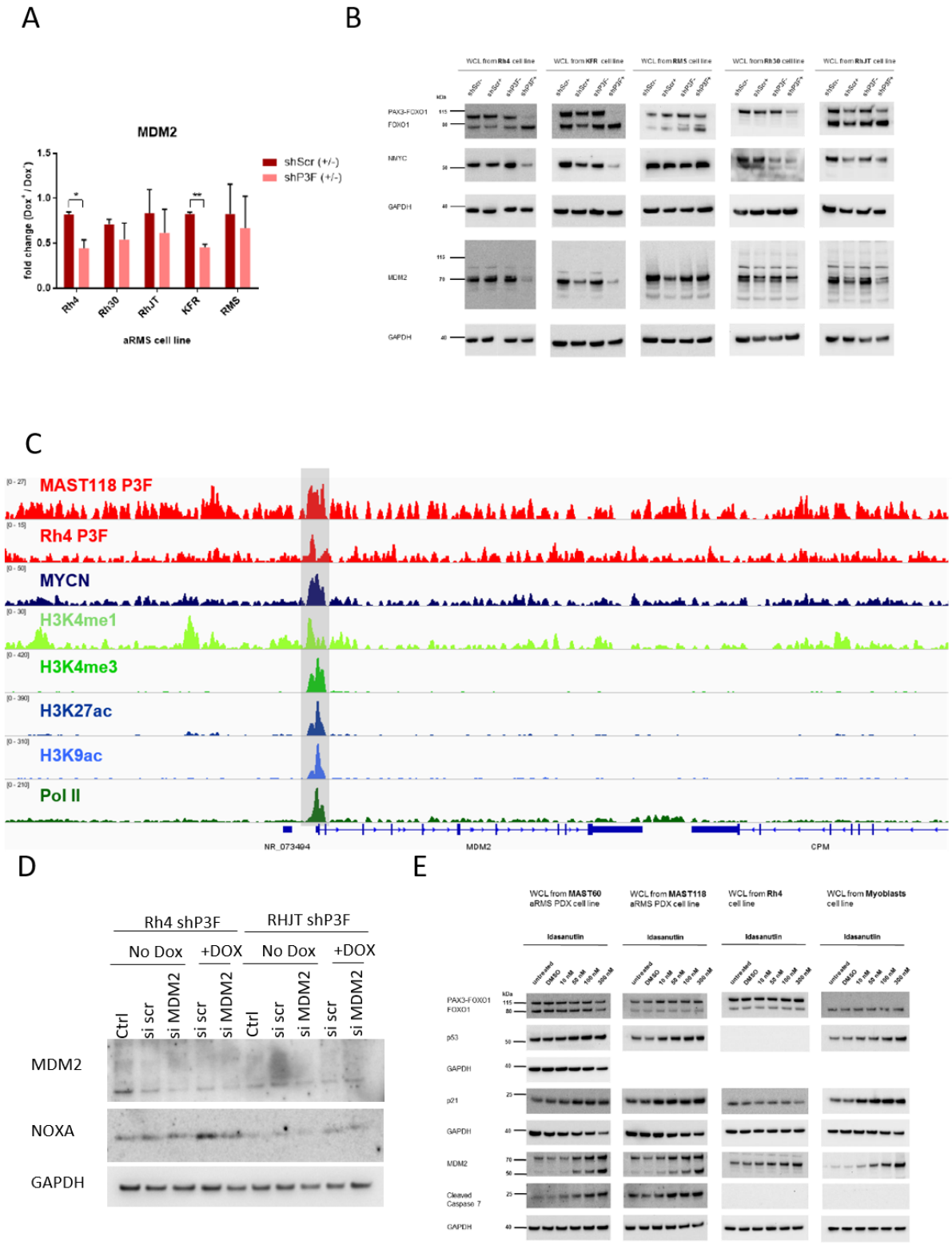
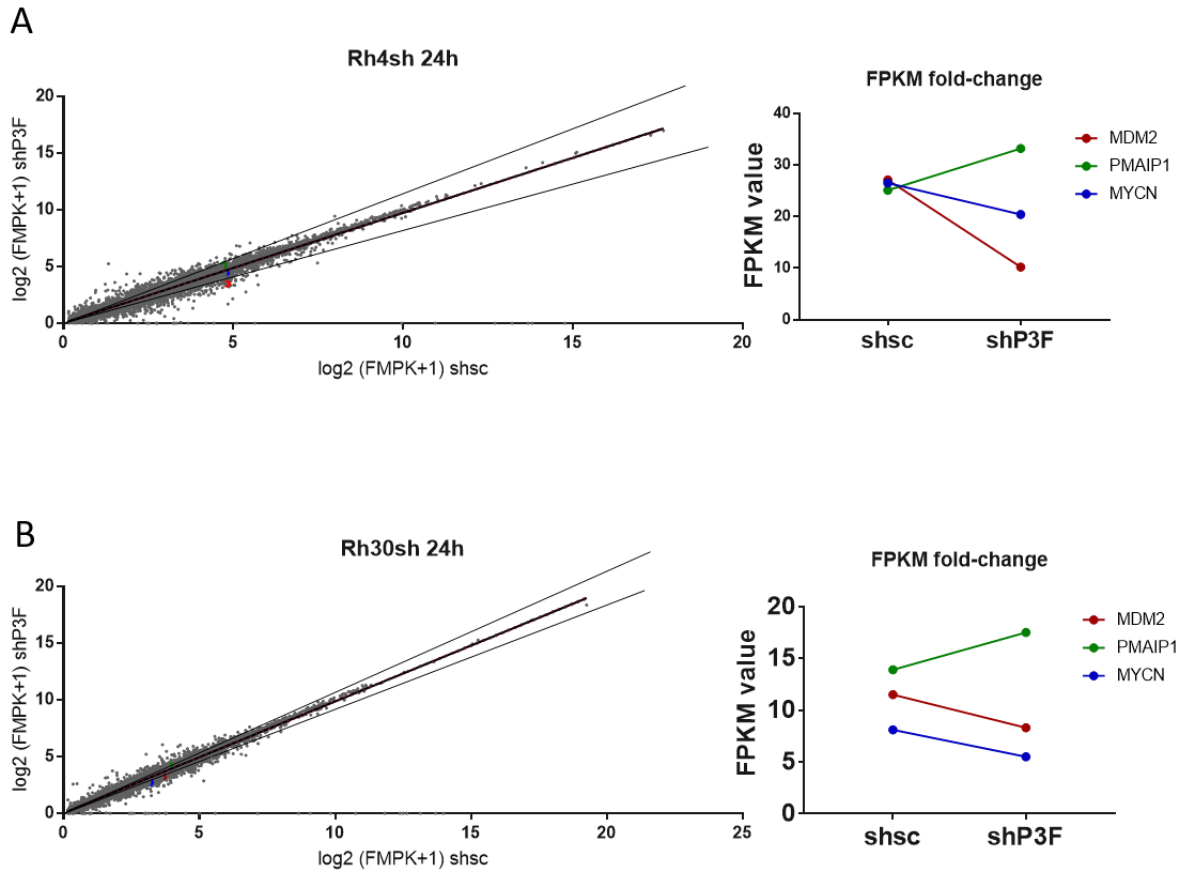

A
MAST118 P3F
Rh4 P3F
MYCN
H3K4me1
H3K4me3
H3K27ac
H3K9ac
Pol II
PMAIP1
B
Rh4 shP3F
siRNA
DOX
Ctrl
scr
FOXO1
N-MYC
PAX3-FOXO1
FOXO1
N-MYC
NOXA
GAPDH
C
RHJT shP3F
Ctrl
scr
FOXO1
N-MYC
D
RH4
Relative Caspase 3/7 activity
Ctrl-
Ctrl+
si scr-
si scr+
si FOXO1-
siFOXO1+
si NMYC-
si NMYC+
sh scr
sh P3F
E
RHJT
Relative Caspase 3/7 activity
*
sh scr
sh P3F

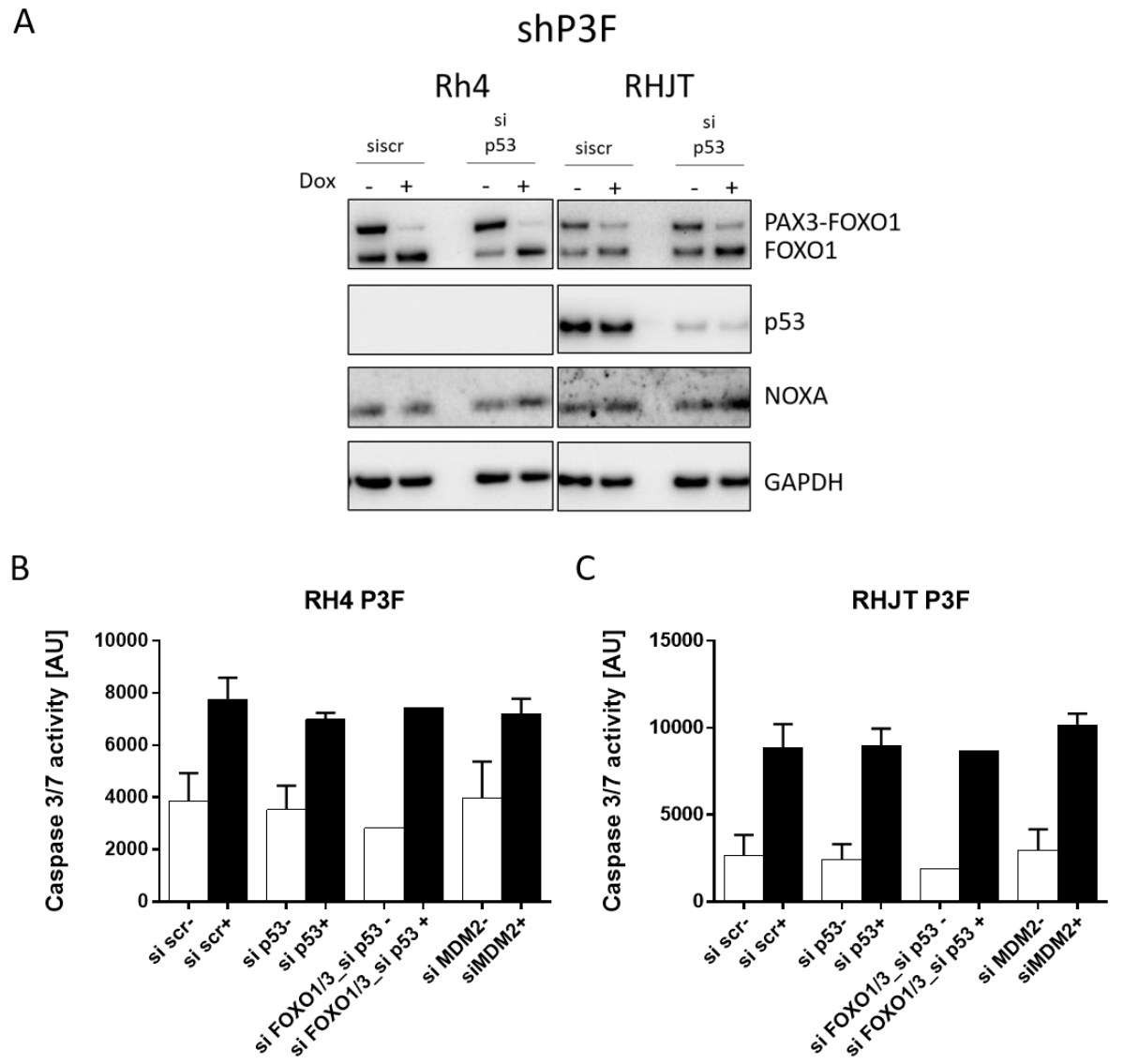
Figure 3


A
MDM2
shScr (+/-)
shP3F (+/-)
fold change (Dox+ / Dox-)
Rh4
Rh30
RhJT
KFR
RMS
aRMS cell line
B
PAX3-FOXO1
FOXO1
MYCN
GAPDH
MDM2
GAPDH
C
MAST118 P3F
Rh4 P3F
MYCN
H3K4me1
H3K4me3
H3K27ac
H3K9ac
Pol II
NR_073494
MDM2
CPM
D
Rh4 shP3F
RHJT shP3F
No Dox
+DOX
Ctrl
si scr
si MDM2
MDM2
NOXA
GAPDH
E
WCL from MAST60 aRMS PDX cell line
WCL from MAST118 aRMS PDX cell line
WCL from Rh4 cell line
WCL from Myoblasts cell line
Idasanutlin
PAX3-FOXO1
FOXO1
p53
GAPDH
p21
GAPDH
MDM2
Cleaved Caspase 7
GAPDH

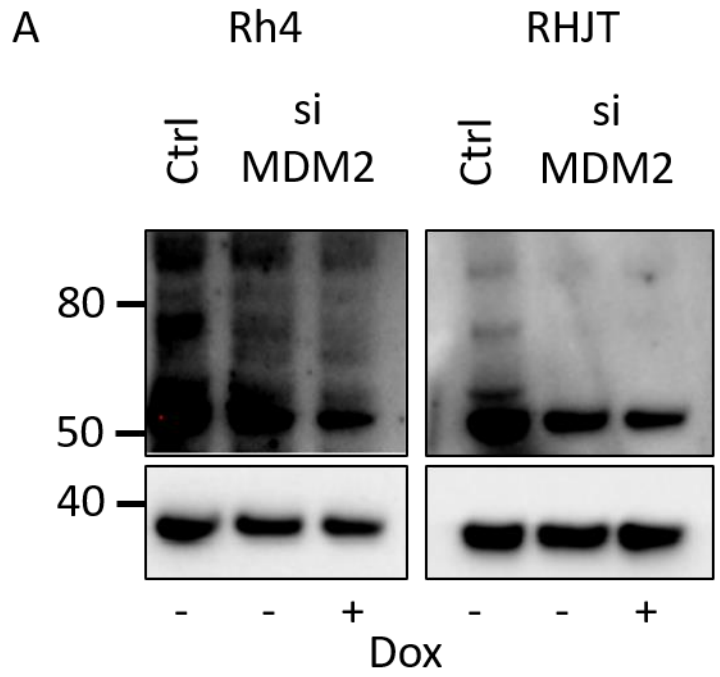
Supplemental Figure 1



Supplemental Figure 2



Supplemental Figure 3



Supplemental Material and Methods

Antibodies

Antigen	Manufacturer	Host (Clone)	Dilution
Cl. CASP7	Cell signaling	rb poly (D198)	1:1000
FOXO1	Santa Cruz	rb poly (H-128)	1:500
GAPDH	Cell signaling	rb mono (14C10)	1:1000
MDM2	Genetex	rb poly (100653)	1:500
MYCN	Cell signaling	rb mono	1:1000
NOXA	Cell signaling	rb mono (D8L7U)	1:1000
P21	Cell signaling	rb mono (12D1)	1:1000
P53	Thermo Fisher Sc	ms mono (DO-1)	1:1000
PARP	Cell signaling	rb poly	1:1000

TaqMan probes

All probes were ordered as Assay-on-Demand mix (Thermo Fisher Scientific) containing specific primers and FAM/BHQ-1 probe.

Gene	Assay ID
PAX3-FOXO1	Hs03024825
GAPDH	Hs02758991_g1
PMAIP1 (NOXA)	Hs00560402
MYCN	Hs00232074
MDM2	Hs01066930

Small interfering RNAs

All siRNAs were ordered from Thermo Fisher Scientific (Cat# 4392420) as lyophilized samples. They were resuspended to a concentration of 5 µmol/L for subsequent experiments.

Target	siRNA Assay ID
FOXO1	s5258
FOXO3a	s5261
MDM2	s8630
MYCN	s526554
TP53	s607
non-targeting (scr)	AM4611

shRNA constructs

In advance we tested three different shP3F sequences targeting the breakpoint region for knockdown efficiency and toxicity and continued with shP3F #3.

Targeting sequences

#1 CCTCTCACCTCAGAATTCAAT
#2 CTCTCACCTCAGAATTCAATT
#3 GGCCTCTCACCTCAGAATTCA

7. DISCUSSION

Optimizing therapy to improve patient outcomes still remains the major challenge in pediatric cancer. Conventional chemotherapy and radiation come with the risk of severe adverse effects for the health and the proper development of children. Insight into the key dependencies of each individual cancer can help tailor new targeted therapy strategies. Our focus of interest is alveolar rhabdomyosarcoma (aRMS), an aggressive cancer with myogenic characteristics. While overall survival of RMS patients increased with the implementation of the VAC regimen, the curve of cure rates has reached a plateau over the last decades, indicating a lack of new therapy options.[84,111] In addition, disseminated aRMS still has an overall 5-year survival of only around 11%.[77] Here, conventional therapy fails and new approaches are desperately needed.

To this end, we aimed to further elucidate the mechanism by which the fusion protein PAX3-FOXO1 prevents cell death in aRMS tumors and to convert this knowledge into a combination therapy approach targeting PAX3-FOXO1 biology and simultaneously priming the cancer cell to a cell death pathway.

7.1 THE ROLE OF NOXA IN PAX3-FOXO1 DEPENDENT CELL DEATH

Using a CRISPR-mediated knockout screen, we demonstrated that, upon loss of PAX3-FOXO1, aRMS cells undergo intrinsic apoptosis that is dependent on NOXA (**M1, Fig.1E**). To underline this finding, another hit in this screen was HUWE1, also known as MCL-1 ubiquitin-ligase E3 (MULE). Depletion of this gene led to a partial rescue from cell death, suggesting an involvement in cell death after loss of PAX3-FOXO1 (**M1, Fig.1E**). However, as the name suggests, HUWE1, or MULE, regulates the anti-apoptotic BCL-2 family member MCL-1 by facilitating its proteasomal degradation.[402] MCL-1 in turn is the canonical inhibitor of NOXA.[153] In our model this would implicate that knockout of HUWE1 leads to higher levels of MCL-1, which would in turn lead to reduced NOXA function, thus preventing the cells from initiating apoptosis after loss of PAX3-FOXO1. We could extend our knowledge on the regulatory mechanisms of cell death by not only identifying the primary pro-apoptotic factor in this context, but, with HUWE1, also identify a potential indirect regulator of this factor. Interestingly, despite this role of the NOXA-MCL-1 axis in our experiments, we demonstrated that not inhibition of MCL-1 most efficiently primed cells for cell death (**M1, Suppl. Fig.2O**), but rather inhibition of BCL-xL. Treatment with ABT-263 proved the most efficient approach. Furthermore, we could demonstrate that the effect of ABT-263 is NOXA-dependent (**M1, Fig. 2G**). Although direct interactions between BCL-xL and NOXA have been described,[197,403] they still remain the exception, suggesting that interaction between NOXA and BCL-xL is not direct. BCL-xL has been described as PAX3-FOXO1 target gene,[144] which would explain the sensitivity of aRMS cells to treatment with ABT-263.

Interestingly, upregulation of MCL-1 has been described as mechanism of ABT-737/ABT-263 resistance in certain cancer types.[404] Thus, we could conclude that the NOXA-dependent mechanism of action of ABT-263 can be counteracted by MCL-1 overexpression, and additional inhibition of MCL-1 might even further enhance the efficiency of ABT-263 treatment. Indeed, inhibition of MCL-1 has been shown to overcome resistance against ABT-263 in cancer cells.[405] In non-small cell lung cancer, a study could show that NOXA displaces and destabilizes MCL-1 and thereby modulates sensitivity of the cells

towards ABT-737.[406] These findings also underscore the importance of NOXA and MCL-1 levels for ABT-263/ABT-737 treatment. In line with these findings, in aRMS, treatment with an mTOR-inhibitor reduced MCL-1 expression and sensitized cells to treatment with ABT-737.[145]

A major drawback of the clinical translation of ABT-263 was the occurrence of severe side effects, like thrombocytopenia in some patients, probably through on-target activity of ABT-263 on BCL-xL.[407,408] Therefore, in our in vivo studies, we regularly assessed weight loss of the mice as an indicator of perturbed well-being of the mice. No increased weight loss, could be observed, indicating that the dosing was well tolerated (**M1, Suppl. Fig. 7B-C**).

Mechanistically, the regulation of NOXA expression appears to be more obscure. We found no evidence for a direct transcriptional regulation of PAX3-FOXO1 on the *PMAIP1* locus (**M2, Fig. 2A**). Indirect regulation can be facilitated through a large variety of factors, which would require large scale unbiased screening approaches for complete coverage. In our experiments we demonstrated that neither MDM2/p53 nor wild-type FOXO1 contribute to NOXA expression. We did find though, that MYCN regulates NOXA expression in aRMS cells (**M2 Fig. 2C-D**) and could confirm MYCN binding to the *PMAIP1* locus by ChIP-Seq data. This finding confirms a recent study showing NOXA upregulation by MYCN in MYCN-amplified neuroblastoma.[409] Furthermore, this regulation confirms a recent finding, that NOXA expression is high in aRMS[147]. Since MYCN is a known target gene of PAX3-FOXO1,[96] paradoxically, MYCN-dependent regulation of NOXA expression would suggest that NOXA expression should decrease upon loss of PAX3-FOXO1 due to concomitant loss of MYCN. However, our observations instead show upregulation of NOXA after silencing of PAX3-FOXO1 (**M2, Fig. 1**). While high basal levels of NOXA in aRMS have already been described[147], our data indicate that there must be other factors involved in NOXA expression which are suppressed under basal conditions by PAX3-FOXO1. As NOXA is a universal stress sensor, there is a plethora of factors, other than p53 and MYCN, known to regulate NOXA expression, for instance HIF-1α, E2F1, c-myc, p73, SalI, ATF3, or c-Jun.[410,411] To unravel the exact mechanism of NOXA regulation after silencing of PAX3-FOXO1 one would need to screen for the potential of each factor individually. In addition, one would need to take into account the innate role in regulating several critical processes that some of these factors possess.

Lastly, in the screen to identify drugs enhancing viability loss after silencing of PAX3-FOXO1 (**M1, Fig.2**), we could observe that not only BH3-mimetics are potent drugs to further sensitize aRMS cells to cell death, but also Aurora kinase A inhibition resulted in loss of viability. PAX3-FOXO1 has been described to be critical for G2/M checkpoint adaptation by which cells can overcome G2/M blockade in case of incomplete DNA damage repair. Aurora kinase A was shown to be essential for checkpoint recovery.[334] Inhibition of Aurora kinase A results in mitotic arrest or senescence.[347] Indeed, we observed an accumulation of cells in G2/M after treatment with the Aurora kinase A inhibitor Alisertib (**M1, Fig. 5F**). Thus, loss of both fusion protein and Aurora kinase A activities potentiate the cell cycle arrest. In this case, untreated control cells would outgrow treated cells resulting in lower viability in the screen readout. Furthermore, Aurora kinase A has been implicated in regulation of anti-apoptotic BCL-2 proteins. Indeed, inhibition of Aurora kinase A has been described to induce downregulation of BCL-xL in cancer cells by affecting signaling,[374] and to shift the Bax/BCL-2 ratio.[375] Such effects, in particular those on BCL-xL activity could explain why Aurora kinase A inhibition sensitizes to NOXA-dependent cell death in our model.

In conclusion, our findings demonstrate the importance of NOXA in cell death after silencing of PAX3-FOXO1, and identify a novel treatment strategy. Treatment efficiency might even be enhanced in MCL-1 low expressing aRMS tumors or with simultaneous pharmacological inhibition of MCL-1, which could also reduce side effects in patients due to lower drug dosing.[412]

7.2 REGULATION OF PAX3-FOXO1 STABILITY BY AURORA KINASE A

In this thesis, we can demonstrate that Aurora kinase A regulates PAX3-FOXO1 stability and that inhibition of the former reduces fusion protein levels in aRMS cells (**M1, Fig.3**).

While we observed reduced PAX3-FOXO1 protein levels with all Aurora kinase A inhibitors tested, the extent of reduction varied significantly. This variation can be explained by different pharmacodynamic parameters (i.e. binding affinity) for each drug. With 100 nmol/L the drug concentrations for the screen were chosen to be on the lower end in order to reduce unspecific effects possibly occurring at high concentrations.

The first question that arose with these results was whether Aurora kinase A exerts direct effects on PAX3-FOXO1 and thus would be a novel putative interactor of the fusion protein. However, co-immunoprecipitation (co-IP) experiments both for PAX3-FOXO1 and for Aurora kinase A showed no interaction between these two proteins (data not shown). Nevertheless, kinase-protein interactions are known to be transient[413] and thus, co-IP protocols might not suffice to capture all interaction partners of Aurora kinase A. Recent mass spectrometric analysis (unpublished results), of interaction partners of PAX3-FOXO1 did not reveal Aurora kinase A as an interaction partner of the fusion protein. In an approach to also capture weak and transient interaction partners, we established the BioID / BirA proximity ligation protocol.[414,415] Here, the bait protein, in our case PAX3-FOXO1, is fused to a bacterial biotin-ligase and incubation of the cells with biotin results in ligation of biotin to any proteins in close proximity of the bait (i.e. interacting proteins). Biotinylated proteins can be purified by streptavidin pull-down. BioID experiments in HEK293T cells, ectopically expressing the PAX3-FOXO1-BioID fusion revealed biotinylation of PLK1 which was already described as an interactor before.[150] Importantly, also Aurora kinase A was biotinylated, indicating that there might indeed be interaction with PAX3-FOXO1. The BioID method however, is prone to errors and heavily relies on adequate controls, as spatial interaction versus direct interaction cannot be distinguished.[416] Thus, our current data does not allow us to distinguish between these possibilities. The lack of Aurora kinase A as a hit in former interaction studies hints more towards a potential indirect regulation of PAX3-FOXO1 by Aurora kinase A.

Aurora kinase A activates PLK1 by phosphorylation at Thr210 (**Figure 13B**). Since PLK1 is known to directly phosphorylate and thereby stabilize PAX3-FOXO1, we assume that at least part of the effect of Aurora kinase A on the fusion protein depends on PLK1 as a downstream target. Indeed, we could observe that inhibition of Aurora kinase A leads to decreased PLK1 phosphorylation at position Thr210. Interestingly, we also found that Aurora kinase A activity correlated with levels of PAX3-FOXO1 phosphorylation at Ser437. This residue so far has not been implicated in fusion protein turnover. The site corresponds to Ser256 in wild-type FOXO1, which is known to be phosphorylated by AKT. In other studies, it could be shown that loss of Aurora kinase A leads to reduction in phosphorylated Ser256 levels of FOXO1, thus linking Aurora kinase A expression or activity to FOXO1 phosphorylation.[417]

These findings are in line with our data on the fusion protein, where Ser437 was less phosphorylated after inhibition of Aurora kinase A.

In wild-type FOXO1, phosphorylation at Ser256 is preceded by acetylation at Lys245 and Lys248, and results in nuclear exclusion and degradation of the protein (**Figure 16A**).[102] Conversely, while acetylation of PAX3-FOXO1 at the corresponding lysine residues Lys426 and Lys429 has been demonstrated, it was shown to stabilize the protein, indicating that acetylation and phosphorylation might have opposite effects both in wild-type FOXO1 and the fusion protein (**Figure 16B**).[104] Along this line of thought, we assumed that following acetylation of the respective residues phosphorylation of Ser437 would lead to stabilization of PAX3-FOXO1, as opposed to the destabilizing effect of Ser256 phosphorylation in wild-type FOXO1. However, this phosphorylation so far has not been studied in PAX3-FOXO1. Here, for the first time, we established that phosphorylation of Ser437 indeed increases fusion protein stability and that lack of this phosphorylation results in decreased stability (**Figure 16B**).

Since in wild-type FOXO1 Ser256 is phosphorylated by AKT, we can assume that AKT also is responsible for phosphorylation of Ser437 in PAX3-FOXO1 (**Figure 16B**). Indeed, in several studies AKT activity was shown to be regulated by Aurora kinase A, and consequently inhibition of the latter led to reduced AKT activity.[418–420] Loss of AKT activity after Aurora kinase A inhibition could thus explain loss of phosphorylation of Ser437 in PAX3-FOXO1 and its decreased stability. Conversely, this mechanism of regulation contradicts data showing that AKT decreases PAX3-FOXO1 activity, albeit at unknown phosphorylation sites.[421,422] Here, further validation of our data is needed to prove that AKT indeed phosphorylates Ser437 and thus enhances fusion protein stability.

Lastly, it cannot be excluded that Ser437 is a potential new interaction site for PLK1. Indeed, phospho-site prediction using the GPS 3.0 tool[423] indicates possible binding of PLKs to that site (data not shown). However, as no further literature exist on PLK1 phosphorylating either wild-type FOXO1 or PAX3-FOXO1, this mode of interaction is less likely.

Recently, we demonstrated that PLK1 phosphorylation at Ser503 protects PAX3-FOXO1 from proteasomal degradation.[150] As for the phosphorylation of Ser437 we so far did not demonstrate a similar mechanism. Here, it would be important to show exactly how Ser437 phosphorylation contributes to fusion protein stability, irrespective of which kinase is responsible for phosphorylation.

In conclusion, we observed that pharmacological inhibition of Aurora kinase A results in reduced PAX3-FOXO1 protein levels, making it an attractive target for precision therapy. On the one hand this was explained by loss of PLK1 activity but also by reduced PAX3-FOXO1 phosphorylation at Ser437, which is another site important for fusion protein stability.

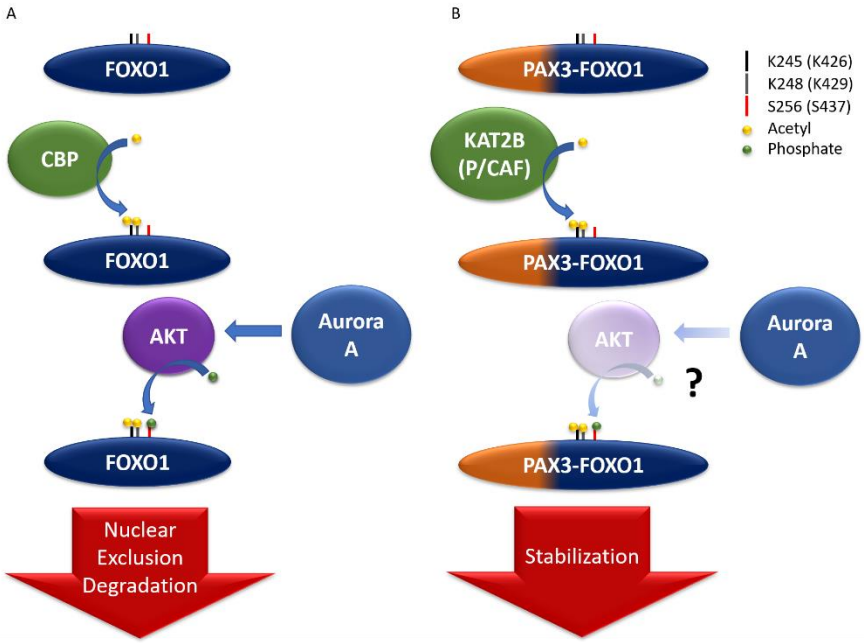


Figure 16: *Regulation of FOXO1 and PAX3-FOXO1 through phosphorylation and acetylation.* ***A)*** *FOXO1 is first acetylated at lysines 245 and 248 which allows for AKT-mediated phosphorylation at serine 256. This results in nuclear exclusion and subsequent degradation.* ***B)*** *PAX3-FOXO1 is also acetylated at the corresponding lysines (K426/K429) which here stabilizes the fusion protein, potentially through Aurora kinase A regulated phosphorylation of serine 437 by AKT (faded purple)*

7.3 THE POTENTIAL OF CDK9 INHIBITION TO INTERFERE WITH PAX3-FOXO1

A second set of hits from the library screen, able to reduce PAX3-FOXO1 protein levels were CDK9 inhibitors. All three CDK9 inhibitors decreased PAX3-FOXO1 protein levels down to approximately 60-75%.

CDK9 is the catalytic core subunit of the positive transcription elongation factor b (P-TEFb). It interacts with the C-terminus of RNA polymerase II, thereby stimulating transcription after initiation.[424] Thus, CDK9 has a critical role in driving gene transcription of a cell.

Our observation that inhibition of CDK9 leads to reduced PAX3-FOXO1 could therefore hint at a transcriptional inhibition of fusion gene expression. So far, information on transcriptional control of PAX3-FOXO1 is sparse. While, regulatory enhancers are known for PAX3 expression, these have not been validated for PAX3-FOXO1.[95] With CDK9 inhibition we would thus have the opportunity to target the fusion protein already at a transcriptional level (**Figure 9B**). However, our findings would first need to be validated in order to see whether reduced CDK9 activity indeed leads to reduced PAX3-FOXO1 expression. The problem here is, that just like epigenetic modifiers such as HDAC inhibitors, the downstream effects would affect a large variety of genes and pathways. This might prove true in particular for CDK9, as it is known to be a universal factor needed for gene transcription. Here it might be difficult to discern potential direct from secondary effects. Secondary effects could comprise decreased expression of direct regulatory factors influencing expressing or stability. Despite many potential off-target effects due the biology of CDK9, the inhibitor Alvocidib has been approved by the FDA as an orphan drug.[425]

Thus, if validated further, CDK9 inhibition might be a potential new strategy to target PAX3-FOXO1 biology through transcriptional downregulation of the fusion gene.

7.4 SYNERGISTIC EFFECTS OF AURORA KINASE A INHIBITION AND BH3-MIMETICS

The effects of Aurora kinase inhibition on cell viability can be explained by loss of PAX3-FOXO1 but in addition there are other factors contributing to this outcome. First, Aurora kinase A regulates MYCN by protecting it from proteasomal degradation (see Fig. 14). MYCN in turn, a potent oncogene, is a known target gene of PAX3-FOXO1[144] and in addition is amplified in 28% of aRMS cases[86], underscoring its importance for this disease. The dependence of tumors on MYCN and the detrimental effects of Aurora kinase A inhibition in that regard have already been established, for instance in MYCN-amplified neuroblastoma.[409] Inhibition of Aurora kinase A thus contributes via several pathways to decreased viability in aRMS tumors. On the one hand, it decreases PAX3-FOXO1 stability, thereby altering MYCN expression. On the other hand, Aurora kinase A inhibition directly targets MYCN stability, thus potentiating the first effect.

Taken together, the high synergy between Aurora kinase A inhibitors and ABT-263 in aRMS can be explained by the several mechanisms by which the combination targets the cancer cell. First, there is loss of PAX3-FOXO1 stability through Aurora kinase A inhibition. Second, as mentioned above, there are direct and indirect effects on MYCN levels, an important oncogene. Third, apart from effects on the oncogene stability, Aurora kinase A inhibition has been shown to induce senescence or mitotic arrest and thus stop cancer cells from proliferating. Loss of PAX3-FOXO1 in this scenario prevents the cell from undergoing G2/M checkpoint adaptation to proceed through the cell cycle. Fourth, ABT-263 has been proposed as a senolytic drug to drive the senescent cells into apoptosis.[260,395] This effect is dependent on BCL-xL which is highly expressed in aRMS cells, as it is a target gene of PAX3-FOXO1.[144] Furthermore, inhibition of Aurora kinase A has been shown to downregulate BCL-xL[374], thus shifting the balance on the mitochondrial membrane even more towards apoptosis and making ABT-263 treatment even more efficient.

These multifaceted mechanism of action makes this combination so potent against fusion-positive aRMS tumors. While the combination of Alisertib and ABT-263 does also show synergy in fusion-negative eRMS cells, it is not as strong as in aRMS cells. This observation can be explained by the combination of mechanisms explained above, which are independent of PAX3-FOXO1. These mechanisms of action can affect cancer cells irrespective of fusion status and thus were expected to have effects. Importantly though, non-cancer cells like fibroblasts or myoblasts were not affected, giving hope for potential therapeutic options with this combination. In addition to that, our in vivo studies showed that overall the combination treatment was well tolerated by the mice.

In conclusion, using Aurora kinase A inhibition and BH3-mimetics, we found a potent combination therapy approach, that shows high synergy in vitro and in vivo.

7.5 THE ROLE OF AURORA KINASE A IN OTHER TRANSCRIPTION FACTOR DRIVEN CANCERS

The importance of Aurora kinase A in sarcomas has only been sparsely investigated. However, there is a clear correlation of Aurora kinase A expression and overall survival in several sarcomas as can be found seen in **Figure 17**. Unfortunately, no such data exists for RMS patients but an equally dire prognosis for Aurora-high patients can be predicted. This estimation is supported by the important role of Aurora A in stabilizing PAX3-FOXO1 and MYCN, which is amplified in almost one third of aRMS cancers[86]. Thus, Aurora kinase A has an important role in aRMS that potentially results in worse prognosis as well and would provide a new target for targeted therapy approaches.

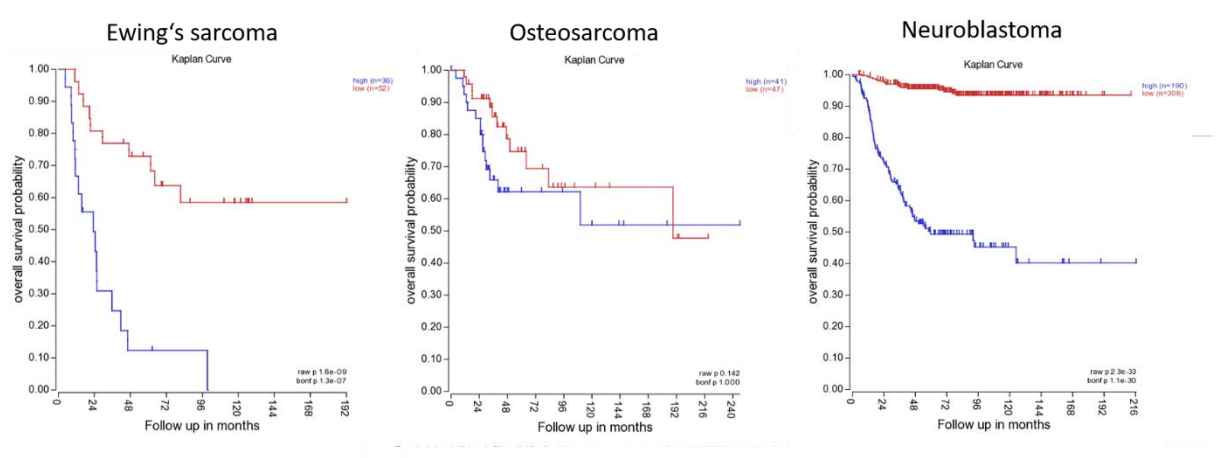


***Figure 17**: Overall survival rates of patients with different pediatric cancers, divided into Aurora kinase A high and low expressing groups. Data obtained from the R2: Genomics Analysis and Visualization Platform (http://r2.amc.nl)*

Figure 17 exemplifies the bad prognosis for Ewing's sarcoma patients if Aurora A is highly expressed. This is particularly interesting, as Ewing's sarcoma, just like aRMS, is also driven by an oncogenic transcription factor (EWS-FLI1), as a result of a chromosomal translocation. Here it would be interesting to establish whether Aurora kinase A could contribute to EWS-FLI1 stability in a similar manner as we demonstrated with PAX3-FOXO1. These insights might reveal general mechanisms how Aurora kinase A contributes to stability of fusion protein transcription factors irrespective of the cancer type. However, such contribution of Aurora kinase A in other sarcomas has not been established so far. In Ewing's sarcoma it could be shown that Aurora kinase A is a target gene of EWS-FLI1 but no direct interaction or effect on stability was demonstrated.[426] Also, in synovial sarcoma, another transcription factor-driven disease, regulation of Aurora kinase A and the fusion protein SS18-SSX has not been investigated so far. Although, as with PAX3-FOXO1 in aRMS, it has been established also here that the fusion proteins are critical for maintenance of their respective sarcomas,[427,428] the pitfalls when targeting a transcription factor remain. Thus, further knowledge of the biology of these oncogenic transcription factors could provide new targets for precision medicine and maybe Aurora kinase A, as potential interactor, could be a worthy option in these cancers.

However, while our data raise hopes for a potential new therapy in children and adolescents with aRMS, numerous past studies show that the clinical efficacy of this approach remains to be demonstrated.

7.6 THE ROLE OF MDM2 AS A POTENTIAL TARGET GENE OF PAX3-FOXO1

Lastly, during our studies, we found hints that MDM2 potentially may be a novel target gene of PAX3-FOXO1. The first indications were derived from transcriptome analyses comparing aRMS cells before and after silencing of PAX3-FOXO1. Here, we observed a reduction of MDM2 expression of around 70% in the PAX3-FOXO1-silenced cells. To validate this finding, we analyzed five different cell lines by qRT-PCR and Western Blot. Indeed, we found that both MDM2 mRNA and protein expression were reduced upon shRNA-mediated silencing of PAX3-FOXO1. Though these findings indicate a regulatory connection between PAX3-FOXO1 and MDM2, they allow no further conclusions whether the regulation is direct or indirect. To further investigate this regulation, we analyzed data from ChIP-Seq experiments. Indeed, in the Rh4 cell line we found PAX3-FOXO1 to be enriched at the promoter region of MDM2. In addition to that, the epigenetic signature at this position hinted at active transcription. However, as this result was only obtained from one cell line, it needs to be validated in additional cells, such as those from PDX material, and functional tests are required to confirm MDM2 as a target gene of PAX3-FOXO1. MDM2 was not connected to NOXA expression in our cell line models. However, most cell lines become deficient for p53 activity or signaling as an adaption to the culture conditions.[429] Typically, aRMS tumors are not deficient for p53 and thus, in primary cell models MDM2 might contribute to NOXA expression.

The importance of MDM2 as an oncogene in RMS to disrupt p53 function is further supported by the fact that amplification of the MDM2 locus is commonly observed in eRMS while p53 is almost never mutated (**Figure 8**).[85] In aRMS PAX3-FOXO1 mediated expression of MDM2 could ensure reduced activity of p53. These findings could indicate that pharmacological disruption of the MDM2/p53 interaction could provide a new means of targeting RMS cells. Already in 2009 a pre-clinical study demonstrated that treatment with Nutlin-3 induced cell death in rhabdomyosarcoma cell lines, supporting this hypothesis.[430] Nutlins are a class of small molecule inhibitors, disrupting the MDM2/p53 interaction, thus stabilizing the tumor suppressor in cancer cells.[431]

In line with these findings, we observed that cells derived from the aRMS patient-derived xenografts MAST60 and MAST118 express wild-type p53. Consequently, treatment with Idasanutlin resulted in increased p53 levels and caspase activity, reflecting their sensitivity towards this mechanism of action. Here, we demonstrate, for the first time, sensitivity of patient-derived aRMS cells to MDM2/p53 disruption. It would be interesting to investigate whether this sensitivity was also given in both Ewing's sarcoma and synovial sarcoma, the other transcription factor-driven sarcomas. Here knowledge of the p53-status could provide new treatment options with Nutlins.

Nutlins and other small molecule antagonists of MDM2 are used in pre-clinical studies and a few early clinical trials give hope that this therapy might in the future progress further into the clinic.[432,433]

In conclusion, we found evidence of a PAX3-FOXO1-dependent regulation of MDM2 expression, though we cannot conclude direct activity of the fusion protein on the MDM2 promoter. Furthermore, we demonstrated for the first time, sensitivity of PDX-derived "primary" aRMS cells to pharmacological MDM2 inhibition.

7.7 CONCLUSION

Overall, our studies presented in this thesis are summarized in the graphical scheme of **Figure 18**. We demonstrate that loss of PAX3-FOXO1 in aRMS cells results in intrinsic apoptosis that is dependent on NOXA. NOXA expression is regulated by MYCN, a target gene of PAX3-FOXO1. Furthermore, we show a novel important role for Aurora kinase A in aRMS cells. Inhibition of Aurora kinase A led to reduced stability of PAX3-FOXO1 facilitated by loss of phosphorylation at Ser437. In accordance with published literature, Aurora kinase A inhibition also reduced MYCN protein stability. In a search for a combination therapy approach, based on our findings, we found that treatment with Alisertib and ABT-263 synergistically induced cell death *in vitro* and with lasting effects delayed tumor growth *in vivo*. Furthermore, we found that primary aRMS cells can also be sensitized to cell death using Idasanutlin. Thus, this thesis sheds light onto how PAX3-FOXO1 biology prevents cell death in aRMS, thereby contributing to malignancy of the disease. In addition, we introduce a novel combination therapy approach in aRMS that might provide new chances in the fight against a dreadful disease of the young.

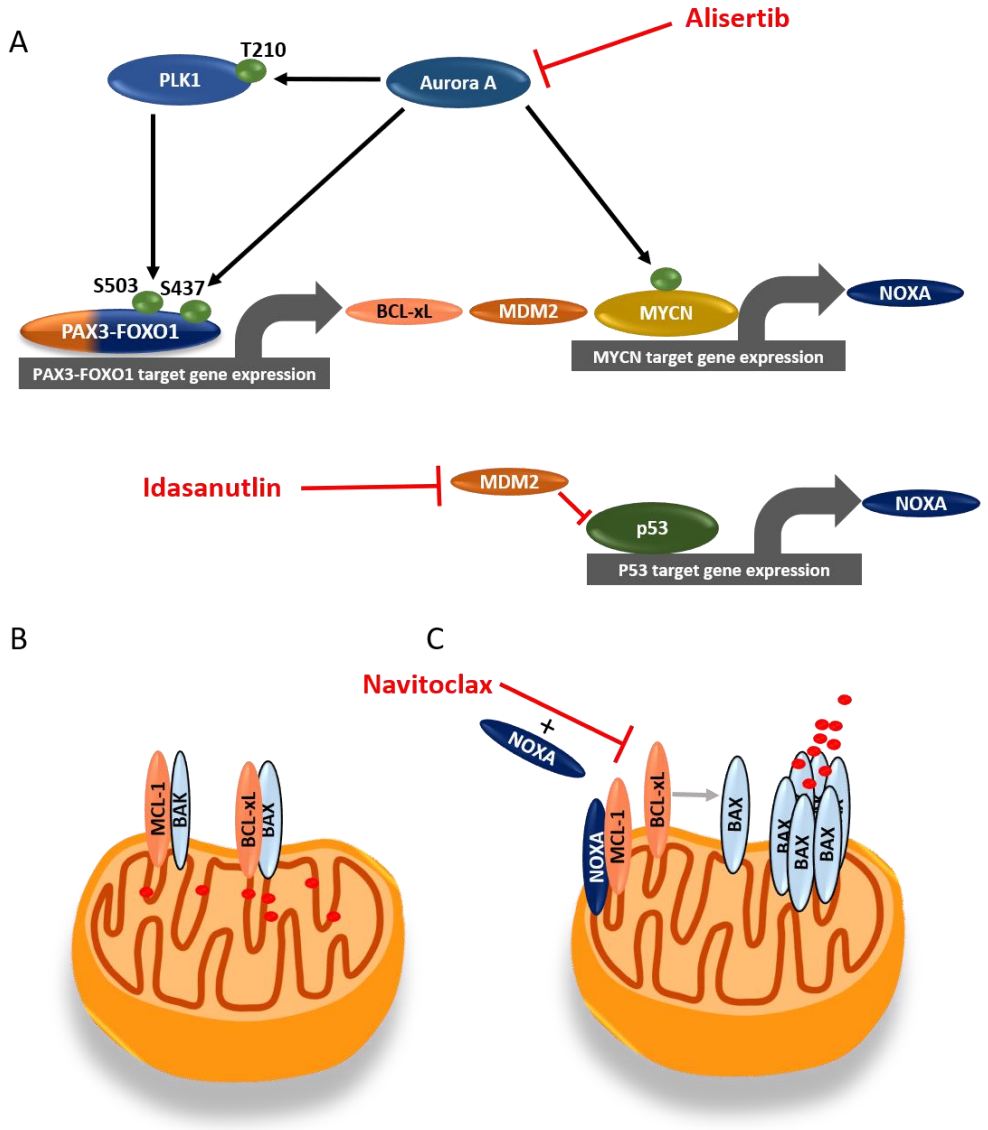


***Figure 18**: **A)** Regulatory network around PAX3-FOXO1 and Aurora kinase A, including transcriptional activity of MCYN on NOXA. **B-C)** Activities of pro-apoptotic BCL-2 proteins (blue) and anti-apoptotic BCL-2 proteins at the mitochondrial membrane. NOXA displaces MCL-1 and Navitoclax can inhibit BCL-xL, allowing for pore formation and release of cytochrome c (red dots) into the cytosol. Red inhibitory arrows indicate pharmacological intervention.*

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9. ACKNOWLEDGEMENTS

"Coming back to where you started is not the same as never leaving"
- Sir Terry Pratchett

Here I am. Almost exactly 10 years ago I started on this scientific journey which led me from Göttingen via Bonn to Zürich. A long time in which so many exciting things happened and shaped my development not only as a scientist but importantly also as a person. I am grateful for all the colleagues, companions, and friends I met along the way. The last 4 ½ years were particularly intense and I would like to thank all these people that contributed to my journey in this time (and apologize if this will be long and cheesy).

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Are you still reading this? You'd better be. Here is the last one but by far not the least. Here is the most precious treasure I found along the way. Here is my beautiful wife, **Andrea**. I simply cannot express in words how much you changed my life and shaped my journey for the better. I am forever grateful for having you by my side, through all the rough times. Thank you for pulling me through them and for brightening up even the darkest day! I love you! It is with you that I will continue the next stages of this journey. Where it might lead us? I do not know. But I am looking forward to it because I will have you by my side and *either road you'll take, I shall go with you.*

10. CURRICULUM VITAE

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Switzerland
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Education

04/2014 – 11/2018

PhD in Cancer Biology
University of Zurich, Switzerland

Thesis: Characterizing oncogene addiction in alveolar rhabdomyosarcoma to find new approaches for combination therapy

Supervisor: Prof. Beat W. Schäfer, Department of Oncology, University Children's Hospital Zurich

09/2011 – 01/2014

Master of Science in Life and Medical Sciences
University of Bonn, Germany

Thesis: The role of MINCLE in sterile inflammation

Supervisor: Prof. Eicke Latz, Institute of Innate Immunity, University of Bonn

Final grade: 1.3*

10/2008 - 08/2011

Bachelor of Science in Molecular Medicine
University of Göttingen, Germany

Thesis: Functional analysis of the androgen receptor and its splice variants in castrate resistant prostate cancer

Supervisor: PD Dr. Paul Thelen, Department of Urology, University Hospital Göttingen

Final grade: 1.7*

** Grading scheme: 1.0 = best / 5.0 = worst*

Training for Biomedical Technical Assistant

08/2007 – 09/2008 Rheinisches Bildungszentrum Köln GmbH, Cologne, Germany

High School

08/1997 – 06/2006 Dietrich Bonhoeffer Gymnasium, Bergisch Gladbach, Germany

Majors: Biology and Chemistry

Work experience

PhD in Cancer Biology

04/2014 – 11/2018 University Children's Hospital Zürich, Switzerland

Student assistant

09/2010 – 08/2011 Department of Molecular Pharmacology, University Hospital Göttingen

Tasks: Construction of lentiviral vectors; reprogramming keratinocytes to induced pluripotent stem cells

Health care assistant

12/2007 – 10/2008 Male nurse, Marienkrankenhaus, Bergisch Gladbach, Germany

Civilian Service

10/2006 – 06/2007 Operation theater nurse at hospital's operating department, Marienkrankenhaus, Bergisch Gladbach, Germany

Work placement

08/2006 – 09/2006 Institute of Tumor Biology, Prof. Klaus Pantel
University Hospital Hamburg-Eppendorf

Volunteer work

Cancer Biology PhD Students' retreat, Emmetten 2017
2017 — Organizing committee for PhD Mini Symposium

7th Students' Symposium of Molecular Biomedicine
2012 — Organizing committee / Bonn, 2012

6th Students' Symposium of Molecular Medicine
2011 — Organizing committee / Göttingen, 2011

YMCA Cologne
2004 - 2009 — Group leader

Selected posters and oral presentations

AACR Annual Conference, Chicago
04/2018 — Poster: Characterizing oncogene addiction in alveolar rhabdomyosarcoma reveals novel strategies for combination therapy

Children's Research Center Symposium, Zürich
11/2017 — Talk: Krebstherapie – Die Mischung macht's

Cancer Network Zürich Retreat
03/2017 — Talk & poster: New strategies for combination therapy in alveolar rhabdomyosarcoma

Children's Research Center Retreat, Zürich
10/2016 — Talk: New strategies for combination therapy in alveolar rhabdomyosarcoma

Award for best oral presentation

EACR Conference – A Matter of Life or Death, Amsterdam
01/2016 — Poster: Characterizing cell death in aRMS following PAX3-FOXO1 silencing reveals novel strategies for combination therapy

3rd place in poster awards